REGULATION OF GERMLINE STEM CELL SURVIVAL AND DNA REPAIR IN THE *DROSOPHILA* TESTIS

by
Salman Hasan

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Abstract

Cells in any organism face significant stresses that can cause damage to their DNA. Sources of DNA damage include endogenous agents such as reactive oxygen species and replication errors or exogenous agents such as exposure to radiation or chemicals known to damage DNA. Ionizing radiation is particularly dangerous for cells because this type of radiation creates DNA double-strand breaks. If unrepaired, these breaks can cause cells to stall during the cell cycle and ultimately die. On the other hand, if repaired incorrectly, harmful mutations can be induced at the site of breaks which can be detrimental to the cell as well as the organism as a whole. Induction of DNA double strand breaks in adult stem cells can be particularly damaging for an organism. Since adult stem cells maintain tissue homeostasis, loss of these cells can lead to collapse of whole tissue systems. Further, induction of DNA double strand breaks in the gamete producing germline stem cells (GSCs) can lead to harmful mutations that may persist in the progeny for generations. Fortunately, studies have shown that multiple adult stem cells are significantly radio-resistant. The causes of this radio-resistance are not very well understood but can be broadly divided in to two categories – 1. Extrinsic signals to stem cells 2. Factors intrinsic to stem cells. In this thesis, we investigate some of the extrinsic and intrinsic factors that can confer nutritional and genotoxic stress-resistance to stem cells in general and GSCs in particular using the adult Drosophila testis niche as a model system. Understanding the factors that preserve genomic integrity and radio-resistance in stem cells can be useful to minimize potential damage to stem cells in tissues in cancer patients who are treated with radio-therapy.

The Drosophila testis niche consists of GSCs that are flanked by somatic stem cells called Cyst stem cells (CySCs). GSCs divide to give rise to differentiating germ cell daughters that ultimately produce sperm. Both GSCs and CySCs attach to a group of non-mitotic cells called the "hub". The hub is known to secrete signals which promote maintenance of the GSCs and CySCs. One such signal is the cytokine Unpaired which activates the Janus kinase-Signal transducer and activator of transcription (JAK-STAT) pathway. Using ionizing radiation, we established that the stem cells in the Drosophila testis are more resistant to low doses of radiation (25 Gy) compared to their differentiating germ cell progeny (spermatogonia) which undergo cell death. The radio-resistance in GSCs was due to the extrinsic signals coming from the hub that locally up-regulate expression of the anti-apoptotic protein Drosophila inhibitor of apoptosis 1 (DIAP1) in stem cells in a JAK-STAT dependent manner. Differentiating germ cells do not receive signals from the hub and are therefore less resistant to ionizing radiation. Similar to ionizing radiation, DIAP1 also protected GSCs from nutritional deprivation stress.

Since ionizing radiation induces double strand breaks in DNA of all cells in the testis apex including the GSCs, long-term survival of the GSCs can only occur with efficient DNA double strand break repair. DNA double strand breaks can be repaired using homologous recombination (HR) or non-homologous end joining (NHEJ). Of the two pathways, NHEJ is faster but more error-prone than HR. In order to study if HR or NHEJ are used by the GSCs to repair their DNA, we established a particular radiation dose of 75 Gy. At this dose, within one day post radiation exposure, half the GSCs are lost in a Chk2, p53 dependent manner. The remaining

half of GSCs are able to regenerate the lost GSCs within a week of radiation exposure. The GSCs have to repair their DNA to successfully divide and regenerate the lost GSCs. By functionally removing different proteins that are required for the HR and the NHEJ repair, we sought to understand the requirement of each of the pathway for GSCs that remained after radiation exposure to successfully regenerate the lost GSCs. Our results showed a requirement for the HR repair pathway in the GSCs that remained post radiation exposure to successfully replenish the GSC pool. Thus our results highlight HR pathway as the cell-intrinsic factor that can promote genomic integrity in GSCs upon radiation exposure. Altogether, this work shows how cell-extrinsic and cell-intrinsic factors can promote survival and renewal of GSCs when faced with stress conditions.

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Chapter 1

Resistance to radiation-induced DNA damage in germline stem cell niches

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Abstract

DNA damage poses significant challenges to cells. Both intrinsic mechanisms, including replication errors and extrinsic mechanisms, such as chemical insults or radiation can induce DNA damage. DNA double-strand break is a type of DNA damage that can be particularly hazardous for cells if unrepaired. Ionizing radiation (IR) is one of the agents that causes DNA double-strand breaks. The repair of these breaks is vital to a cell's survival and a highly conserved molecular pathway serves to detect and repair these breaks. While most cells in an adult organism are thought to sense DNA double-strand breaks using this conserved repair pathway, the downstream events, including the type of repair used, can be cell-type specific. This tissue-specificity in whether and how DNA damage is repaired extends to stem cells, which support the long-term renewal of many adult tissues. While some somatic stem cells are radiosensitive and others are not, gamete-producing germline stem cells (GSCs), which reside within specialized local microenvironment in the gonads of a wide range of organisms are highly radio-resistant compared to their differentiating progeny across species. Here, we summarize the current literature regarding the cellular and molecular responses of adult GSCs within the well-characterized *Drosophila melanogaster* (fruit fly) testis stem cell niche, with comparisons to GSCs in the adult fly ovary, the *C. elegans* gonad and the mouse *(M. Musculus)* testis. In all the three systems, the existence of evolutionarily conserved signaling mechanisms including signals from the niche, or from adjacent dying cells, promote radio-resistance in GSCs but not in differentiating germ cells. The response of stem cells and their niches to IR-induced DNA damage is broadly relevant because it could

facilitate our understanding of how radiation used to treat cancer patients may affect genome integrity, survival and function of GSCs more generally.

Introduction

Germline stem cells (GSCs) have the unique ability to self-renew and produce daughter cells that differentiate into eggs or sperm (reviewed in Spradling et al., 2011). GSCs are found in the adult testes of most male organisms and in the adult ovaries of the well-studied model organism *Drosophila melanogaster* (fruit flies), but they have not been clearly identified in the adult ovaries of mammals (reviewed in Dunlop et al., 2014; Spradling et al., 2011). They also sustain gametogenesis in the *C. elegans* hermaphrodite and male gonads (reviewed in Kimble and Seidel, 2013). Like other adult stem cells, GSCs are found in niches (local micro-environments) that maintain their fate. In contrast to all other adult stem cells, GSCs are uniquely responsible for gamete production. While the number of GSCs declines with age in flies, worms and rodents, these stem cells continue to function even in older animals (reviewed in Schultz and Sinclair, 2016). They are also very resistant to environmental stresses such as protein starvation. A well-studied example is the response of GSCs in the adult fruit-fly testis and ovary to temporary protein deprivation, which causes a reversible loss of GSCs (reviewed in deCuevas and Matunis 2011). Similarly, GSCs are specifically maintained in the adult hermaphrodite worm during prolonged starvation, and regenerate the tissue when nutrients are restored (Angelo and Van Gilst, 2009). Here, we focus on the ability of GSCs in Drosophila, *C. elegans* and *M. Musculus* to respond to one particular type of stress: the genotoxic stress caused by IR.

IR induces DNA double-strand breaks. Of all the types of DNA damage this is the most consequential with regard to cell survival, as these breaks must be repaired

before a cell can replicate its DNA and divide (reviewed in Santivasi and Xia, 2014). Although the responses of most tissue-specific adult stem cells to DNA damage in general, and to IR-induced damage specifically are not well characterized, there are a few examples where this has been studied in detail, revealing tissue-specific outcomes. For example, in the adult mouse, intestinal stem cells are more radiosensitive than both hematopoietic stem cells (HSCs) and hair follicle bulge stem cells. Many, but not all, adult somatic stem cells are radio-resistant, when compared across different tissues. For instance, melanocyte stem cells in adult mice are more radiosensitive than hair follicle bulge stem cells and differentiate massively and are lost from the niche in response to low doses of IR (Inomata et al., 2009; Sotiropoulou et al., 2010). Massive differentiation of melanocyte stem cells in response to IR is thought to occur due to reduced self-renewal and increased differentiation of these stem cells (Inomata et al., 2009). Many types of adult stem cells are also more resistant to IR than their differentiating progeny. This includes HSCs (Mohrin et al., 2010a), hair follicle bulge stem cells (Sotiropoulou et al., 2010), and keratinocyte stem cells (Rachidi et al., 2007). However, the role of the stem cell niche in promoting stem cell resistance to IR is not well-understood. Here, we discuss recent work showing that GSCs in the adult fly testis niche are more resistant to IR-induced cell death than to their differentiating progeny, and we relate these findings to studies from germline stem cells in the fly ovary, the worm (*C. elegans*) gonad and the mouse (*M. Musculus*) testis.

Adult GSCs are more resistant to cell death than their differentiating progeny

The process of gamete production from GSCs in adult gonads is largely conserved across species. GSCs are found adjacent to the somatic cells in the gonads. The somatic cells form the GSC niche, which secretes the signals that maintain and promote the survival of the GSCs (reviewed in Greenspan et al., 2015; Kimble and Seidel, 2013; Wu et al., 2016). GSCs divide asymmetrically, producing daughters that remain in the niche (self-renewal) and daughters that are displaced from the niche and differentiate (reviewed in Greenspan et al., 2015). Differentiating germ cell progeny can undergo either a few or very many rounds of mitosis with incomplete cytokinesis, forming clusters of interconnected germ cells called spermatogonia in males, cystocytes in females (reviewed in Greenspan et al., 2015) and mitotic germ cells in *C. elegans* hermaphrodites (reviewed in Hubbard, 2007). These interconnected germ cells undergo meiosis and continue to differentiate, ultimately forming mature sperm or eggs.

The ability of germ cells in adult organisms to survive after exposure to IR has been examined in many species, and in all cases, GSCs differ in their ability to survive in comparison to their differentiating progeny (spermatogonia) (Hasan et al., 2015; reviewed in Shetty and Meistrich, 2005) or cystocytes (Xing et al., 2015). For example, when adult fly testes are examined after high doses of IR and stained with terminal dUTP nick end labeling (TUNEL) to identify cells undergoing apoptosis, many dying germ cells can be found outside the niche but none can be found within the niche (Hasan et al., 2015; Xing et al., 2015), suggesting that GSCs are more resistant than spermatogonia to IR-induced cell death. Similar observations have

been made in this system using other markers for cell death (Xing et al., 2015), a range of IR doses, and at different time points after exposure to IR (Welshons and Russel, 1957). They have also been made in other systems including the adult fly ovary (Wylie et al., 2014; Xing et al., 2015), the adult hermaphrodite worm gonad (Gartner et al., 2008), and the testes of adult mice (Rübe et al., 2011; van der Meer et al., 1992) and rats (Albuquerque et al., 2013; Erickson, 1976; Withers et al., 1974). Together, these observations indicate that GSCs in adult gonads are more resistant to IR than their differentiating progeny. Combined with the knowledge of the responses of the adult stem cell to IR in the mammalian skin and blood mentioned previously, these observations suggest that resistance to IR is a feature common to many types of stem cells. Interestingly, this ability does not extend to all adult stem cell populations. As mentioned above, Melanocyte stem cells and Intestinal stem cells are lost by differentiation and death, respectively, in response to IR (reviewed in Blanpain et al., 2011). One reason for radio-sensitivity in melanocyte and intestinal stem cells could be reduced expression of anti-apoptotic and DNA repair proteins (reviewed in Blanpain et al., 2011). Whether this radio-sensitivity is an intrinsic property of the stem cells, is imposed by differences in niche signaling, or both, is not known.

In addition to their resistance to irradiation, GSCs also appear to be more resistant to cell death than their differentiating progeny under normal physiological conditions. For example, in adult male flies, dying GSCs are rarely seen in any study (Hasan et al., 2015; Yacobi-Sharon et al., 2013), but 20-30% of spermatogonia routinely die before they complete spermatogenesis (Hasan et al., 2015; Yacobi-

sharon et al., 2013). Similar observations have been made in the adult fly ovary; dying GSCs are rarely seen, but about 2% of differentiating germ cell clusters in each ovariole (which are the functional units of the ovary) are undergoing cell death (Drummond-Barbosa and Spradling, 2001). Dying differentiating germ cells are also a common feature of adult rodent testes (0.21% of spermatogonia in each tubule die) (Rodriguez et al., 1997). Similarly in *C. elegans,* about half of the germ cells die over the lifetime of the adult hermaphrodite (Gumienny et al., 1999). As in the fly testis, dying GSCs are rarely detectable in worm gonads and rodent testes when cell death is examined using TUNEL or morphological criteria for cell death such as DNA condensation under normal physiological condition (reviewed in de Rooij and Grootegoed, 1998; Gartner et al., 2008). The function of physiological germ cell death in all of these cases is speculative, but could serve to eliminate germ cells that are defective in completing meiosis because they harbor unresolved DNA double - strand breaks or chromosomal abnormalities that prevent successful chromosomal synapsis during meiosis (reviewed in Shaha et al., 2010).

The molecular mechanisms driving physiological germ cell death have been studied extensively in the adult hermaphrodite worm and in the Drosophila ovary (reviewed in Bailly and Gartner, 2013; Pritchett et al., 2009). Caspases mediate canonical apoptosis in the germ cells in the adult fly ovary (reviewed in Pritchett et al., 2009) and in adult hermaphrodite worms (Gumienny et al., 1999). Interestingly, germ cell death in the adult fly testis differs from that in the ovary. Germ cell death in the fly testis exhibits morphological features of both apoptosis and necrosis (Yacobi-Sharon et al., 2013). Germ cell death differs molecularly from somatic cell death in

this case: it does not involve effector caspases (Hasan et al., 2015; Yacobi-Sharon et al., 2013). In an interesting parallel, germ cell death in the adult rodent testis also exhibits features of both apoptosis and necrosis (Allan et al., 1992) and is thought to occur using a non-apoptotic alternative cell death pathway (Knudson et al., 1995; Rodriguez et al., 1997). The use of caspase-independent pathways for germ cell death in the adult fly testis could be due to a subsequent-non-apoptotic role for these enzymes in differentiating germ cells: caspases are required for spermatid individualization in Drosophila (Arama et al., 2003). A similar role of caspases has been identified in spermatid individualization in adult mice (reviewed in Fuchs and Steller, 2011), suggesting a high degree of conservation. Caspase inhibitors are being considered as therapy for neurodegenerative diseases and in organ transplants (reviewed in Callus and Vaux, 2006). Therefore, it would be interesting to know if human spermatid individualization process also requires caspases, since side effects of therapies involving caspase inhibition could include undesired effects on human fertility (reviewed in Steinhauer, 2015).

GSC resistance to IR is mediated by niche signaling

The fact that GSCs are more resistant to IR-induced cell death than their differentiating progeny suggests that signals from the niche could be contributing to their survival. The clearest evidence supporting this hypothesis comes from studies of the fly testis. The fly testis niche contains two populations of stem cells, sperm-producing GSCs and adjacent somatic stem cells which give rise to the somatic cyst cells that encase and support the differentiation of germ cells outside the niche. Many

signaling pathways promote the maintenance of fly testis GSCs and somatic stem cells (reviewed in de Cuevas and Matunis, 2011b) including the highly conserved Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway (Kiger et al., 2001; Tulina and Matunis, 2001). One of the targets of JAK-STAT signaling is the gene encoding the conserved anti-apoptotic protein Death-associated inhibitor of apoptosis 1 (DIAP1) (Betz et al., 2008b; Hasan et al., 2015). Expression of DIAP1 is upregulated in GSCs and somatic stem cells relative to spermatogonia. Loss of DIAP1 results in rapid loss of GSCs or somatic stem cells both under normal conditions and following exposure to IR, revealing that DIAP1 is necessary for maintenance of both stem cell lineages in this tissue (Hasan et al., 2015). As expected, because DIAP1 is a target of JAK-STAT signaling, GSCs in testes with reduced JAK-STAT signaling are also more susceptible to death following exposure to IR; significantly more dying GSCs are detected in these testes than in control testes exposed to the same dose of IR (Hasan et al., 2015). Under normal conditions, however, GSCs in testes with reduced JAK-STAT signaling differentiate but do not die (Brawley and Matunis, 2004). The most likely explanation is that GSCs with reduced JAK-STAT signaling produce enough DIAP1 to protect them from death under normal conditions, but cannot withstand the extra stress induced via IR. Together, these observations support a model in which signals from the niche promote death resistance in GSCs by up-regulating the conserved anti-apoptotic protein DIAP1.

Differentiating germ cells, which have left the niche, could be more susceptible to cell death because they no longer receive the signals that protect the

GSCs. Therefore, in the fly testis, one might expect that ectopic up-regulation of DIAP1 in differentiating germ cells would be sufficient to rescue them from death, but this is not the case; ectopic up-regulation of DIAP1 in Drosophila spermatogonia does not rescue them from normal or IR-induced cell death. Unexpectedly, these cells are rescued non-autonomously from IR-induced death by ectopic upregulation of DIAP1 in adjacent somatic cyst cells (Hasan et al., 2015). This finding suggests that some spermatogonia die after exposure to IR because their accompanying cyst cells die. Upregulation of DIAP1 in cyst cells does not rescue spermatogonia from death under normal physiological conditions, however, suggesting that cyst cell death is not the main cause of spermatogonial cell death under normal conditions in Drosophila (Hasan et al., 2015).

Niche signaling is also likely to promote the survival of adult GSCs in mice. The principal niche signal in adult mouse testes, glial cell line-derived neurotrophic factor (GDNF), is required for GSC maintenance in vivo (Meng et al., 2000). GDNF also promotes GSC maintenance in vitro, where it has been shown to activate the Serine/Threonine kinase Akt in GSCs (Oatley et al., 2007). Activation of Akt is known to promote protein synthesis, survival and cell proliferation via the conserved protein mammalian target of rapamycin (mTOR) (reviewed in Hemmings and Restuccia, 2012). Blocking Akt signaling causes a significant increase in the number of apoptotic cells in self-renewing germ cell cultures and a significant decline in the number of functional GSCs in these cultures, as assayed functionally by transplantation into recipient testes (Oatley et al., 2007). Although the role of the Akt pathway has not been tested in GSCs in vivo, it is likely that GDNF promotes GSC

survival in vivo by up-regulating anti-apoptotic factors via the Akt pathway. In other mouse tissues, like adult mouse cardiomyocytes, transgenic expression of Akt has been shown to promote resistance to apoptosis after ischemia-reperfusion injury (Fujio et al., 2000). Akt plays a similar anti-apoptotic role in *C. elegans* germ cells. *C. elegans* null for the *akt* gene display an increase in germ cell death following exposure to IR compared to wild-type controls. Conversely, IR-induced germ cell death is reduced by globally expressed gain of function mutations in *akt* (Quevedo et al., 2007). However, whether niche signaling promotes the activation of Akt specifically in the anterior most germ cells in hermaphrodite worms, which are thought to be the GSCs, is not known (Rosu and Cohen-Fix, 2017). Unlike worms and mice, loss of Akt does not lead to germ cell death in either adult fruit-fly testis or ovary (Roth et al., 2012; Vachias et al., 2014).

Taken together, evidence across the mammalian, worm and fruit-fly model systems seem to suggest that promotion of the expression of anti-apoptotic factors in GSCs by niche signals could be an evolutionarily conserved mechanism that ensure survival and maintenance of GSCs under variety of adverse conditions including ionizing radiation.

Signals from dying germ cells contribute to IR resistance in GSCs

Extrinsic signals that promote GSC resistance to IR can come from somatic niche cells and also from dying germ cells outside the niche. For example, in the adult fly ovary, after exposure to IR, dying differentiating germ cell clusters in the germarium release the ligand PDGF and VEGF related factor 1 (Pvf1), which activates the

receptor tyrosine kinase Tie in nearby GSCs (Xing et al., 2015). Tie activation is not required in GSCs under normal conditions, but it protects IR-exposed GSCs from death by blocking expression of the pro-apoptotic gene *hid*. In *tie* mutant females that are exposed to IR, GSCs are rapidly lost, presumably because they fail to repress *hid* and die by apoptosis. When Pvf1 is knocked down in somatic niche cells before the flies are exposed to IR, the GSCs survive as well as those in control flies; however, when Pvf1 is knocked down in differentiating germ cells, IR-exposed GSCs are lost from the niche. These data support the hypothesis that Pvf1 emanating from IR-exposed differentiating germ cells, rather than niche cells, promotes GSC survival via activating Tie in GSCs. A similar mechanism protects epithelial cells in the fly larvae from death; when cells in one region of the larval wing disc are induced to die (by exposure to IR or by genetic methods), neighboring cells are protected from death by Pvf1-mediated activation of Tie (Bilak et al., 2014). A similar phenomenon may also occur in the fly testis; differentiating germ cells that die in response to nutritional (protein) starvation are also thought to promote the survival of nearby GSCs. However, the signals that come from the dying germ cells in this case are not known (Yang and Yamashita, 2015).

In adult mice, apoptotic cells in liver have been found to secrete mitogens that affect neighboring cells, promoting proliferation (reviewed in Fuchs and Steller, 2015). On the other hand, apoptotic cells in the hair follicle of adult mice secrete death factors that cause additional cell death (reviewed in Fuchs and Steller, 2015). If signals are released by dying germ cells in mammalian testes, and whether or not these include signals that promote the survival of GSCs, is not known.

In addition to a large body of work using dying Drosophila or mice somatic tissues mentioned above, there is also precedent for signals from dying cells that promotes autophagic clearance of the cellular corpse. For example, In *C. elegans* embryo, dying somatic cells elicit phosphatidylserine signals that lead to engulfment of the corpse by nearby somatic cells (Y.-Z. Chen et al., 2013).

Taken together, data from different model systems suggests that dying cells can send multiple paracrine signals to neighboring cells especially in the case of germ cells. Exploration of these signals can help us understand the process of recovery and regeneration in injured tissues.

Intrinsic responses to IR in GSCs and differentiating germ cells

IR causes double-strand breaks in DNA, which must be repaired for the cell to survive. In the canonical DNA damage sensing pathway, cells detect DNA double-strand breaks through a highly conserved pathway that leads to the phosphorylation of the Histone 2A variant H2AX (H2AvD in flies). The phosphorylation of H2AX triggers a signaling cascade that ultimately activates the tumor suppressor p53, resulting in cell cycle arrest and giving the cell time to repair the double-strand breaks. If the damage cannot be repaired, then the cell undergoes programmed cell death (reviewed in Goodarzi and Jeggo, 2013). DNA damage response pathways are evolutionarily conserved and germ cell responses to DNA double-strand breaks induced by IR have been studied in fruit-flies, worms and mice. In female flies that have been exposed to IR, phosphorylated H2AvD becomes evident in both GSCs and their differentiating progeny, but a reporter for p53 activity is thought to be

upregulated specifically in GSCs (Wylie et al., 2014). The same is true in testes from IR-exposed flies and in fly ovaries with genetically engineered double-strand breaks. However, the mechanism(s) limiting p53 activation are not understood.

After DNA double-strand breaks are detected, they are typically repaired by one of two major mechanisms: homologous recombination (HR) or non-homologous end joining (NHEJ) (reviewed in Chapman et al., 2012). HR requires a homologous copy of the damaged DNA as a template for the repair and can proceed only in S or G2 phases of the cell cycle. HR involves several proteins. Chief among them is Rad51 which is necessary for the strand invasion step in order to faithfully repair the double-strand break (reviewed in Jasin and Rothstein, 2013). NHEJ does not use a template and is therefore more error-prone than HR, but it has the advantage of being functional at any phase of the cell cycle (reviewed in Lieber, 2010). It employs the Ku70/80 proteins along with DNA ligase IV and DNA dependent protein kinase catalytic subunit (DNA-PKcs) to process and fix double-strand breaks (reviewed in Lieber, 2010). HR and NHEJ repair pathways can compete. For example, the tumor suppressor protein breast cancer 1 (BRCA1) promotes HR over NHEJ, while p53 binding protein 1 (53BP1) promotes NHEJ over HR (reviewed in Chapman et al., 2012).

In fly ovaries and testes, all GSCs are actively cycling and spend the majority of their time in S or G2 phase (Hsu et al., 2008; Sheng and Matunis, 2011), so they have the potential to use either type of repair mechanism (HR or NHEJ). Using a reporter system to analyze the repair of genetically induced DNA double-strand breaks, Preston et al. found that male germ cells use both types of repair mechanisms,

but the relative use of each pathway changes dramatically as the flies age, with a shift from NHEJ in young males to HR in old males (Preston et al., 2006). However, the promoter used by Preston et al. to express their reporter was later found to be expressed in differentiating meiotic germ cells (spermatocytes), rather than in GSCs (Lu et al., 2013), suggesting that the repair events measured were not occurring in GSCs. Therefore, the repair pathways used by GSCs and whether they change over time, or if they differ from the pathways used by the differentiating germ cells, remains an open question.

The relative contributions of NHEJ and HR have also been studied in *C. elegans*. Germ cells in larval stages of development in hermaphrodite worms use HR and not NHEJ to repair IR-induced DNA double-strand breaks (Clejan et al., 2006). Hermaphrodite larvae with RNA interference mediated knockdown of HR or NHEJ pathway members in germ cells were irradiated. The irradiated larvae were allowed to grow into adults and their progeny were assayed for fertility defects. Irradiated larvae lacking HR pathway components in germ cells developed into adult that were sterile or gave rise to sterile progeny, whereas irradiated larvae lacking an NHEJ pathway member in germ cells yielded fertile adults, similar to irradiated wild-type larvae (Clejan et al., 2006). It remains to be seen if this is true for GSCs in the adult fruit-fly ovary and testes. It is also possible that there are specific niche signals that ensure that the GSCs preferentially use HR to repair the DNA double-strand breaks, by promoting the expression of HR pathway members and restricting the expression of NHEJ members.

DNA damage sensing and repair have also been examined in several different adult mammalian stem cell systems, as mentioned above. In hematopoietic and hair follicle stem cells, as in fly GSCs, anti-apoptotic proteins from B cell lymphoma (Bcl) 2 family such as Bcl2, Bcl-xl etc. and p53 are rapidly and strongly upregulated after IR exposure (Mohrin et al., 2010a; Sotiropoulou et al., 2010). Unlike fly GSCs, however, these somatic stem cell populations are relatively quiescent; most of the stem cells are not cycling at the time of exposure and therefore unable to use HR repair (Mohrin et al., 2010a; Sotiropoulou et al., 2010). Instead, both use NHEJ, which allows for robust repair of the IR-induced DNA double-strand breaks but is associated with the acquisition of mutations and genomic rearrangements (reviewed in Helleday et al., 2014). These results suggest that quiescent stem cells, contrary to what one might expect, are intrinsically more vulnerable than actively cycling cells to mutagenesis as a consequence of DNA damage repair after IR exposure (Mohrin et al., 2010a; Sotiropoulou et al., 2010).

Since mutations in the GSCs have the potential to damage even the next generation, it is possible that GSCs use a different strategy than somatic stem cells to repair IR-induced DNA damage. Rube et al. (2011) examined DNA double-strand break repair in GSCs from IR-exposed adult male mice. By comparing repair success (measured as loss of 53BP1 foci) in control mice to that in mice deficient for DNA-PKcs, they found that GSCs in the mouse testis do not depend on the canonical NHEJ pathway involving DNA-PKcs but instead sense and repair DNA double-strand breaks by a form of NHEJ that is independent of DNA-PKcs called alternative-NHEJ (Rübe et al., 2011). Alternative NHEJ is known to be slower and more mutagenic

than canonical NHEJ (reviewed in Dueva and Iliakis, 2013). Indeed, the DNA-PKcs independent NHEJ repair used by GSCs in adult mice testis was found to be slower than the canonical DNA-PKcs dependent NHEJ operating in other adult mice somatic tissues such as lung, kidney and brain, as assayed by the slower rate at which foci of phosphorylated H2AX are cleared from the cells after IR exposure (Rübe et al., 2011). It is not clear why the more mutagenic alternative NHEJ is evolutionarily preferred over canonical NHEJ in adult mice GSCs (Rübe et al., 2011). It is also unclear whether some or all of the GSCs in an adult testis are actively traversing the cell cycle, which would affect repair pathway choice.

Male mice also have additional mechanisms for preventing the production of mutated sperm. Vrooman et al., observed an elimination of male germ cells harboring meiotic errors by cell cycle checkpoint mechanisms in the spermatocyte stages in adult mice (Vrooman et al., 2014) leading to low mutation frequency in mature spermatozoa. These findings suggest that there are mechanisms to eliminate germ cells with defects that impact meiosis such as unresolved DNA double-strand breaks or improper pairing among sex chromosomes.

GSC resistance to IR is conserved in humans

In men, exposure to IR can result in temporary or permanent sterility. Doses of IR that are high enough to kill all germ cells result in permanent sterility, but after exposure to lower doses, fertility can be restored over time (reviewed in Meistrich, 2013). This phenomenon is thought to occur when doses of IR are high enough to kill differentiating germ cells but not GSCs. For example in one study, a dose of

0.2Gy IR administered to the testis in adult men resulted in 80% loss of differentiating germ cells and 66% reduction in sperm count compared to pre-radiation exposure levels. However, sperm count was restored to pre-exposure levels in 80 weeks by the surviving GSCs (Clifton and Bremner, 1983). This data suggests that GSCs are more resistant to IR-induced cell death than their differentiating progeny and can replenish the supply of sperm after exposure to IR (reviewed in Meistrich, 2013). The mechanisms that promote IR resistance in human GSCs are not known, but could be similar to those used in other organisms. In women, exposure to IR depletes the pool of reserve oocytes, causing premature menopause and infertility. Older women can be impacted more than younger women as the number of reserve oocytes declines with age (reviewed in Kort et al., 2014). Lost oocytes are not replaced and fertility is not regained over time, consistent with the idea that there are no active or potential GSCs in young or older women. Permanent loss of ovarian function has also been observed in children who have been exposed to IR (Green et al., 2009). Temporary or permanent sterility can be a problem for male and female patients who are undergoing IR therapy for cancer, both when the IR is directed specifically at their gonads and also when it is directed elsewhere in the lower abdominal region (reviewed in Kort et al., 2014). For example, spermatogenesis can be severely affected as a result of IR therapy for Hodgkin's lymphoma, which is a cancer of the lymphatic system (Bujan et al., 2014). Currently, gonadal shielding by lead is used to reduce radiation damage to spermatogenesis during IR-based cancer treatment in the abdominal region of the body (reviewed in Kort et al., 2014). Cryopreservation of sperm before cancer treatment is currently the

best therapeutic procedure to preserve fertility of men undergoing treatment. The cryopreserved sperm can then be used in intrauterine or in-vitro fertilization procedure depending on the quality and quantity of sperm collected (reviewed in Osterberg et al., 2014). However, maintenance of fertility through cryopreservation is costly. Additionally, sperm cryopreservation is not a viable option for preserving fertility in pre-pubertal boys (reviewed in Kort et al., 2014). Hormonal suppression of testosterone post IR has been tested as a means to preserve fertility post cancer treatment using IR (reviewed in Meistrich and Shetty, 2008). In a study on adult rats, a 96% reduction in testosterone levels can be induced by injecting Gonadotrophic releasing hormone (GnRH) antagonist to the testis post IR. This treatment reduced apoptosis in spermatogonia by 50% compared to vehicle controls (Shuttlesworth et al., 2000). Additionally, primary spermatocytes were visible in testosterone suppressed testes at 5 weeks post IR, while in controls primary spermatocytes did not reappear until 21 weeks post IR (Shuttlesworth et al., 2000). Testosterone suppression in rats post IR is hypothesized to promote the ability of somatic cells in the testes to maintain and promote differentiation of spermatogonia, however the exact molecular basis for this phenotype is not well understood (reviewed in Meistrich and Shetty, 2008). While results from testosterone suppression in rats were positive, testosterone suppression was found to be ineffective in clinical trials in adult men undergoing radiotherapy in reducing time for recovery of fertility (reviewed in Meistrich and Shetty, 2008), highlighting the need to develop new, cost effective therapies to preserve fertility in patients undergoing radiotherapy. Understanding niche signals and factors intrinsic to GSCs that promote survival of GSCs post IR can

be used to develop new strategies to maintain fertility in patients. Dedifferentiation of spermatogonia to GSCs suggests that spermatogonia have the capacity to respond to niche signals (Barroca et al., 2009). The ability to inject drugs or small molecules that mimic niche signals into the testes of patients undergoing IR treatment to promote anti-apoptotic signaling and DNA damage repair in spermatogonia could promote germ cell survival post IR.

Lessons from GSCs can be applied to the study of cancer stem cells (CSCs)

Cancer stem cells (CSCs) are specialized cells with stem cell-like properties that are thought to self-renew and produce daughters that form the bulk of tumor masses (reviewed in Nassar and Blanpain, 2016). CSCs have been found in tumors from many types of cancer including leukemia and glioblastoma (reviewed in Chen et al., 2013). Like many other adult stem cells, CSCs can survive chemo- and radio-therapy better than their progeny (in this case, other tumor cells) (reviewed in Abdullah and Chow, 2013). Surviving CSCs are thought to be responsible for the regrowth of tumor masses after therapy, thereby giving rise to cancer relapses (reviewed in Nassar and Blanpain, 2016).

Niche signaling is thought to affect CSCs as it affects normal stem cells, by providing signals that promote maintenance and survival of CSCs (reviewed in Nassar and Blanpain, 2016); for example in the adult mouse brain, perivascular niche signals activate proliferative pathways such as Notch and Hedgehog signaling pathways in glioma cancer stem cells (reviewed in Codrici et al., 2016). CSC niches are also thought to provide protection to CSCs in niches subjected to chemo or

radiotherapy (reviewed by Wicha et al., 2006). The mechanisms by which niches promote radio-resistance in CSCs could be similar to the ones that promote IR-resistance in GSCs; for example enhanced expression of anti-apoptotic proteins in CSCs induced by niche signals (reviewed in Wicha et al., 2006) or expression of ATP-binding cassette (ABC) transporters in the CSCs confer multi-drug resistance (reviewed in Wicha et al., 2006). Therefore, the GSC niche can act as a model system for studying mechanisms of IR resistance in cancer and perhaps for identifying signaling pathways that could be targets for chemo-therapy in CSCs.

Conclusion

GSC resistance to IR stress is a conserved phenotype across organisms. Although gonads from different species vary greatly in morphological complexity, GSCs are remarkably similar in their ability to survive doses of IR that are fatal for differentiating germ cells. The higher radio-resistance in GSCs compared to differentiating germ cells is medically relevant in multiple instances. It can be utilized for regeneration of spermatogenesis from surviving GSCs to reduce infertility arising from IR exposure during cancer therapy. The germ cell niche can also act as a model system to understand the pathways promoting survival of CSCs during radio-therapy. Lastly, GSC resistance to IR appears to be an evolutionarily conserved phenomenon with relevance not only to the basic biology of adult stem cells but also to the biology of CSCs.

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Chapter 2

Niche signaling promotes stem cell survival in the Drosophila testis via the JAK-STAT target DIAP1

This chapter is a modified version of the manuscript - Hasan, S., Hetie, P., Matunis, E.L., 2015. Niche signaling promotes stem cell survival in the Drosophila testis via the JAK-STAT target DIAP1. *Dev. Biol. 404, 27–39*.

Abstract

Tissue-specific stem cells are thought to resist environmental insults better than their differentiating progeny, but this resistance varies from one tissue to another, and the underlying mechanisms are not well-understood. Here, we use the Drosophila testis as a model system to study the regulation of cell death within an intact niche. This niche contains sperm-producing germline stem cells (GSCs) and accompanying somatic cyst stem cells (or CySCs). Although many signals are known to promote stem cell self-renewal in this tissue, including the highly conserved JAK-STAT pathway, the response of these stem cells to potential death-inducing signals, and factors promoting stem cell survival, have not been characterized. Here we find that both GSCs and CySCs resist cell death better than their differentiating progeny, under normal laboratory conditions and in response to potential death-inducing stimuli such as irradiation or starvation. To ask what might be promoting stem cell survival, we characterized the role of the anti-apoptotic gene Drosophila inhibitor of apoptosis 1 (*DIAP1)* in testis stem cells. DIAP1 protein is enriched in the GSCs and CySCs and is a JAK-STAT target. *DIAP1* is necessary for survival of both GSCs and CySCs, and ectopic up-regulation of DIAP1 in somatic cyst cells is sufficient to non-autonomously rescue stress-induced cell death in adjacent differentiating germ cells (spermatogonia). Altogether, our results show that niche signals can promote stem cell survival by up-regulation of highly conserved anti-apoptotic proteins, and suggest that this strategy may underlie the ability of stem cells to resist death more generally.

Introduction

Adult stem cells maintain tissues by producing both stem cells and differentiated daughters that replenish lost or dying cells to ensure the integrity of the entire organism (reviewed in Rossi et al., 2008). Stem cells reside in specific microenvironments termed niches, which produce signals that maintain stem cell populations (reviewed in Jones and Wagers, 2008; Spradling et al., 2001). All cells within an organism, including stem cells, can be challenged by cellular stresses that lead to cell death (reviewed in Sancar et al., 2004). These stresses can arise endogenously, such as from the accumulation of reactive oxygen species or errors during DNA replication, or exogenously from environmental insults including radiation or poor nutrition (reviewed in Drummond-Barbosa and Spradling, 2001; Sancar et al., 2004).

In general, stem cells are thought to be more resistant to cell death than their differentiating progeny (reviewed in Mandal et al., 2011). However, this is not the case for all stem cells. The response of stem cells and their progeny to ionizing radiation as a source of stress has been well characterized for several adult mammalian tissues (reviewed in Blanpain et al., 2011b). While stem cells in some tissues are more radio-resistant than their differentiating progeny, stem cells in other tissues are highly susceptible to terminal differentiation or death upon ionizing radiation (reviewed in Blanpain et al., 2011b; Liu et al., 2014). For example, Hematopoietic stem cells (HSCs), mammary stem cells, hair follicle bulge stem cells (BSCs) (Sotiropoulou et al., 2010) and keratinocyte stem cells in the adult mouse are much more radio-resistant than their differentiating progeny (reviewed in Liu et al.,

2014; Rachidi et al., 2007). In contrast, melanocyte stem cells, which reside in the same niche as BSCs, are not radio-resistant. The same dose of radiation that is tolerated by BSCs induces massive terminal differentiation in melanocyte stem cells (reviewed in Blanpain et al., 2011b; Inomata et al., 2009; Insinga et al., 2014; Sotiropoulou et al., 2010). Mammalian intestinal stem cells (ISCs) are also extremely sensitive to radiation: radiation doses lower than those tolerated by BSCs induce ISC death (reviewed in Blanpain et al., 2011b; Sotiropoulou et al., 2010). In cases of radio-sensitive stem cells, radiation-induced DNA damage triggers the highly conserved process of programmed cell death (PCD).

Programmed cell death (PCD) occurs via three canonical pathways: necrosis, autophagy and apoptosis (reviewed in Fuchs and Steller, 2011). Necrosis involves swelling and rupture of the cellular organelles and is characterized by an increase in intracellular Ca^{2+}, reactive oxygen species and acidity (reviewed in McCall, 2010). Autophagy involves engulfment of subcellular components by autophagosomes, which fuse with lysosomes to form autophagolysosomes where the engulfed cytoplasmic material is degraded (reviewed in Fuchs and Steller, 2011). Apoptosis (or type I PCD), the most common and well-studied form of PCD, is characterized by cell shrinkage, nuclear condensation and membrane blebbing. Apoptosis involves the activation of cysteine proteases called caspases (reviewed in Kerr et al., 1972; Ouyang et al., 2012). Initiator caspases are activated in response to apoptotic stimuli, and in turn, they cleave and activate effector caspases, which triggers the cell death cascade (reviewed in Fuchs and Steller, 2011).

Apoptosis is tightly regulated, and highly conserved anti-apoptotic proteins serve to block this typically irreversible process. This includes the inhibitor of apoptosis (IAP) and the B cell lymphoma 2 (Bcl-2) families of proteins, which inhibit caspase activation, thereby preventing their unwanted activity in the absence of death inducing stimuli (reviewed in Fuchs and Steller, 2011; Ryoo and Baehrecke, 2010). Anti-apoptotic proteins are widely expressed and required, and increased expression of anti-apoptotic proteins in cells can confer resistance to apoptotic stimuli such as ionizing radiation-induced DNA damage (reviewed in Liu et al., 2014). Indeed, it has been observed that radio-resistant stem cells such as HSCs have an enhanced expression of Bcl-2 (an anti-apoptotic member of the Bcl-2 family) compared to their daughters as studied in cell culture assays (Mohrin et al., 2010a). In contrast, radio-sensitive ISCs have reduced expression of Bcl-2 when compared to radio-resistant, Bcl-2 enriched, colon stem cells as observed by immunohistochemistry for Bcl-2 in the murine small intestine (reviewed in Liu et al., 2014; Merritt et al., 1995). However, not much is known about why stem cells in certain tissues have enhanced expression of anti-apoptotic proteins. There has been some recent evidence that Sox2, a transcription factor responsible for neural stem cell (NSC) self-renewal, also regulates expression of the IAP family member Survivin, which prevents NSC apoptosis under normal laboratory conditions (Feng et al., 2013). These data suggest that signals from the stem cell niche may cause an up-regulation of anti-apoptotic proteins specifically in stem cells, thereby making them cell death resistant.

Owing to its simplistic arrangement and the abundance of tools for genetic manipulations, the Drosophila testis niche is a useful model system to study the

relation of stem cell survival to niche signaling. The Drosophila stem cell niche resides in the testis apex and consists of a cluster of quiescent niche cells called the hub (Fig. 2.1A). Two types of stem cells, the germline stem cells (GSCs) and the somatic cyst stem cells (CySCs), surround and adhere to the hub (Fig. 2.1A) (reviewed in de Cuevas and Matunis, 2011b; Matunis et al., 2012). The GSCs divide to give rise to differentiating daughter cells called gonialblasts, which divide to produce 2, 4, 8 and 16 cell interconnected spermatogonial cysts (Fig. 2.1A). The spermatogonia further differentiate into spermatocytes, which undergo meiosis to give rise to 64 sperm. The 4 and 8 cell spermatogonial clusters have high levels of the differentiating factor Bag of Marbles (Bam) when compared to GSCs and early daughters (Fig. 2.1A). Two CySCs envelop each GSC, and are required for the survival and proper differentiation of the germ cells. The nuclei of the CySCs are displaced from the hub relative to the nuclei of the GSCs. The CySCs divide to produce daughter cells called cyst cells. These cyst cells no longer divide, but instead elongate and differentiate as they encase the differentiating germ cells.

The hub produces various niche signals that are necessary for stem cell maintenance (reviewed in de Cuevas and Matunis, 2011b). One such signal is the ligand Unpaired (Upd), which binds to its receptor in both the GSCs and CySCs, activating the Janus kinase/ Signal transducer and activator of transcription (JAK-STAT) pathway in these cells (Kiger et al., 2001; reviewed in Stine and Matunis, 2013b; Tulina and Matunis, 2001). Activated STAT leads to the transcriptional up-regulation of genes within the stem cells that are important for their maintenance, while differentiating cells are located away from the hub and presumably receive less

of the Upd signal. GSCs and CySCs require JAK and its downstream target STAT to prevent their differentiation (Flaherty et al., 2010; Hombría and Brown, 2002; Kiger et al., 2001; Leatherman and Dinardo, 2008; Ma et al., 2014; Stine et al., 2014; Tulina and Matunis, 2001). However, whether niche signals promote stem cell viability in this tissue has not been investigated.

Previous studies of cell death in the adult Drosophila testis have focused on dying differentiating germ cells (spermatogonia), which are readily detected in both Drosophila and mammalian testes. On average, 20-30% of differentiating spermatogonia die under normal laboratory conditions in every fly testis, even in testes from young adult males (Yacobi-Sharon et al., 2013); a similar percentage of spermatogonia also undergo cell death spontaneously in rodent testes (Allan et al., 1992). In both organisms, the type of germ cell death differs from canonical apoptosis as it includes morphological characteristics of both necrosis and apoptosis (Allan et al., 1992; Yacobi-Sharon et al., 2013). This includes cellular acidification and reactive oxygen species accumulation as seen in necrosis, as well as cellular shrinkage and chromatin condensation, which are characteristic of apoptosis (Yacobi-Sharon et al., 2013). Studies of stem cell death in both rodents and fly testes have suggested that the GSCs are more resistant to stress-induced death compared to their differentiating progeny (Dym and Clermont, 1970; Erickson, 1976; Ishii et al., 2014a; Welshons and Russel, 1957). However, it is unclear how the GSCs are able to survive stress conditions which are lethal for their differentiating progeny. Here we investigate the occurrence of cell death within the GSCs and CySCs under normal laboratory conditions and stress-induced conditions, and determine whether niche

signals and anti-apoptotic genes (IAPs) play a role in stem cell survival within the Drosophila testis niche.

Results

Stem cells are resistant to normal and stress-induced cell death

To determine whether stem cells, like spermatogonia, frequently undergo cell death under normal laboratory conditions, we sought to quantify and compare the number of dying stem cells and differentiating cells within the adult Drosophila testis. Dying cells in this tissue are identifiable through terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining; however, since TUNEL-positive cells are in advanced stages of dying (Gavrieli et al., 1992), they typically have lost expression of cell-type specific antigens (Darzynkiewicz et al., 1997). Thus, it is not straightforward to establish the identity of TUNEL-positive cells in many cases. Therefore, we focused our efforts on two zones based on testis morphology: the stem cell zone and the differentiating cell zone. A DNA counterstain was used to identify all nuclei, and the stem cell zone included all nuclei within the first two tiers from the hub (Fig. 2.1A). Since TUNEL-positive GSCs and CySCs are indistinguishable, but nuclei from the latter are displaced slightly further from the hub, selecting two tiers ensured the inclusion of all stem cells. It also meant that gonialblasts and early cyst cells were occasionally included in the stem cell zone, but this did not affect our results since TUNEL-positive cells were not detected within the stem cell zone under normal laboratory conditions (N= 25 testes) (Fig. 2.1E and F). In comparison, dying germline cysts were readily detected in the differentiating cell zone, which included all nuclei immediately proximal to the stem cell zone to the end of the region of the testis containing Bam-positive spermatogonia (8-16 cell spermatogonial stage, Fig. 2.1A). On average, 3.32 TUNEL-positive spots per testis were seen in the differentiating cell zone in controls (N= 25 testes) (Fig.2.1E and F).

Since each testis in this genetic background contains an average of 16 spermatogonial cysts (Sheng et al., 2009), we estimate that 20.75 % of the spermatogonial cysts are dying, which is consistent with the previously published estimate of 20% (Yacobi-Sharon et al., 2013). If GSCs die at a similar rate, we would expect to see 2 TUNEL-positive spots per testis within the stem cell zone since each testis in this genotype contains an average of 10 GSCs. However, we did not observe any dying cells in the stem cell zone in this genotype (N= 25 testes) (Fig. 2.1E and F), which is a statistically significant difference (p= 0.0001, Chi-square test). Dying cells were never seen in the stem cell zone when flies were kept under standard laboratory conditions in all genotypes examined in this study (N=177 testes). Similarly, if CySCs were dying, we should have seen an even higher number of TUNEL-positive spots in the stem cell zone. Since we did not observe any dying cells in the stem cell zone, we conclude that stem cells in the adult Drosophila testis do not undergo cell death as often as their differentiating progeny when flies are kept under standard laboratory conditions.

The lack of dying stem cells in testes from well-fed young males suggested that stem cells are more resistant to death than spermatogonia. To pursue this further, we next asked whether stem cells and their daughters could differentially survive various environmental stresses. Since adult stem cells in some tissues are resistant to γ-irradiation (IR), which causes DNA double-strand breaks (DSBs) that result in apoptosis if unrepaired (reviewed in Roos and Kaina, 2006), we chose to characterize the effects of IR on cells within the testis niche. Historical data suggested that stem cells but not differentiating cells survive 4000 Roentgens (or approximately 34 Gy) of

ionizing radiation (Welshons and Russel, 1957). Therefore, we irradiated young adult males with 25 or 50 Gy of IR, and then examined the testis apex for the presence of TUNEL-positive spots. A significant increase in dying spermatogonial cysts (as measured by TUNEL-positive spots within the differentiating zone) was seen in testes from flies exposed to 25 Gy IR compared to un-irradiated controls (from 3.32 to 7.35 spots, $p<0.01$) (Fig. 2.1B, C and E), but no increase in cell death was observed in the stem cell zone (Fig. 2.1E). In contrast, exposure to 50 Gy of IR caused a significant increase in dying cells in both the stem cell ($p=0.048$) and differentiating cell ($p<0.001$) zones compared to un-irradiated controls (with 3% and 64% of the stem cells and spermatogonia, respectively, becoming TUNEL-positive) (Fig. 2.1E). We conclude that the increased resistance of stem cells in the Drosophila testis to cell death relative to their differentiating progeny applies to both normal laboratory conditions and irradiation conditions (25 Gy).

To confirm and extend these results we repeated our irradiation experiment (Fig. 2.1E) but analyzed testes with antisera against the cleaved (activated) effector caspase death caspase 1 (Dcp-1). In the ovary, this antibody labels nurse cells in dying egg chambers (Sarkissian et al., 2014). Since activation of Dcp-1 precedes fragmentation of DNA, marked by TUNEL, co-staining with anti-Dcp-1 and cell-specific antibodies can identify dying cells (Sarkissian et al., 2014). As expected, very few cleaved Dcp-1 positive cells were detected in wild-type control testes. Co-staining with anti-Traffic Jam (TJ) antisera to highlight CySC and cyst cell nuclei revealed an average of 0.1 CySCs and 0.65 cleaved Dcp-1 positive cyst cells per testis apex (N=20) (Fig S2.1A, C). Since Dcp-1 is not required for the spontaneous

spermatogonial cell death that occurs in flies kept under standard lab conditions (Yacobi-Sharon et al., 2013), we considered it likely that the male germline may not express this antigen. Indeed, cleaved Dcp-1 positive germ cells were never seen in control testes (N=20) (Fig. S2.1A). Upon 25 Gy of IR, as expected the number of cleaved Dcp-1 positive GSCs and CySCs was unchanged (an average of 0 GSCs and 0.2 CySCs per testis apex, N=20) (Fig. S2.1B, C) and the number of cleaved Dcp-1 positive cyst cells increased to an average of 1.75 per testis apex compared to 0.65 in un-irradiated controls (Fig. S2.1B, C). This is consistent with our results obtained with TUNEL staining and taken together, these results indicate that stem cells are more resistant to death under normal conditions and upon irradiation.

We next asked whether stem cells in the Drosophila testis are also resistant to milder, more physiologically relevant stresses, such as protein starvation. Protein starvation causes germline and somatic cells to die in the Drosophila ovary (Drummond-Barbosa and Spradling, 2001; Pritchett et al., 2009a). Protein starvation also causes the number of GSCs to decrease due to differentiation in the testis, but its effects on cell death were not quantified (McLeod et al., 2010; Sheng and Matunis, 2011). Therefore, we placed flies on a protein starvation diet for 19.5 hours, and then assayed dying cells in the testis using TUNEL labeling. This dietary change caused a significant increase in cell death in the differentiating cell zone but not in the stem cell zone compared to un-starved controls (Fig. 2.1B, D and F). We conclude that the ability of stem cells in the Drosophila testis to resist death better than their differentiating progeny is seen both under normal laboratory conditions, under

extreme stress such as IR, and under more moderate stress such as temporary protein starvation.

JAK-STAT signaling by niche cells causes DIAP1 enrichment in stem cells and their immediate daughters

To understand why stem cells in the Drosophila testis are more resistant to cell death than their differentiating progeny, we focused on the anti-apoptotic factor Drosophila inhibitor of apoptosis 1 (DIAP1). DIAP1, encoded by the essential gene *thread* (Hay et al., 1995), is the best characterized IAP (Inhibitor of Apoptosis) family protein found in flies. DIAP1 binds to and inhibits the initiator caspase Dronc and the effector caspases DrICE and Dcp-1 (reviewed in Meier et al., 2000b; Wang et al., 1999; Wilson et al., 2002), and blocks apoptosis during normal development and after irradiation (Betz et al., 2008; Meier et al., 2000a; Ou et al., 2003). Expression of pro-apoptotic proteins like Reaper, Grim or Hid (Head involution defective) can inhibit DIAP1 and cause cell death (Chai et al., 2003; reviewed in Fuchs and Steller, 2011). Both GSCs and CySCs can be forced to die by overexpression of Hid and Grim respectively (Hetie et al., 2014; Lim and Fuller, 2012, Greenspan and Matunis unpublished data). Immunostaining young adult testes with anti-DIAP1 antisera revealed that DIAP1 is weakly expressed throughout the testis and is enriched in both GSCs and the CySCs relative to their differentiating progeny (Fig. 2.2A, A').

The enrichment of DIAP1 in the stem cells of the Drosophila testis suggests that niche signals may be regulating *DIAP1* expression. JAK-STAT signaling is a major niche signaling pathway in the Drosophila testis and the single Drosophila

STAT, STAT92E, is locally activated within GSCs and CySCs to promote their maintenance (Brawley and Matunis, 2004; Kiger et al., 2001; Leatherman and Dinardo, 2008). *DIAP1* is transcriptionally up-regulated by JAK-STAT signaling in the Drosophila wing imaginal disc (Betz et al., 2008a), suggesting it could be a STAT target in the testis apex as well. To determine if *DIAP1* is transcriptionally regulated by STAT in GSCs and CySCs, we used established procedures to either reduce or increase JAK-STAT signaling in the testis (Issigonis et al., 2009; Issigonis and Matunis, 2012; Stine et al., 2014), then assayed for changes in DIAP1 levels soon thereafter, prior to stem cell loss. To reduce JAK-STAT signaling, we shifted adult flies carrying a temperature sensitive allele of *STAT92E* to non-permissive temperature for 24 hours. We observed a substantial decrease in DIAP1 levels in all cells within the testis apex, especially cells closest to the hub (Fig. 2.2B, B'). Quantification of DIAP1 expression levels within the stem cells revealed a significant decrease in protein expression when compared to controls (Fig. 2.2D). To increase JAK-STAT signaling, we ectopically expressed the JAK-STAT ligand *unpaired* briefly throughout the testis. This caused an up-regulation of DIAP1 protein in all cells within the testis apex, including stem cells and their differentiating progeny (Fig. 2.2C, C'). Quantification of DIAP1 levels specifically in GSCs and CySCs from these testes revealed a statistically significant increase in DIAP1 expression levels compared to control testes (Fig. 2.2E). Together, these results indicate that DIAP1 is a direct or indirect target of JAK-STAT signaling in the Drosophila testis, and support the hypothesis that the increased expression of DIAP1 within the stem cells compared

to their differentiating progeny seen in wild-type control testes is due to niche signaling.

DIAP1 is necessary for stem cell survival

Because the stem cells of the Drosophila testis are enriched in DIAP1 and are highly stress-resistant, we next wanted to investigate DIAP1 function in these cells. DIAP1 is best characterized as an anti-apoptotic protein (Hay et al., 1995), but also has non-apoptotic roles, including regulating border cell migration during oogenesis (Geisbrecht and Montell, 2004). We used two independent approaches to determine the requirement for DIAP1 in GSCs and CySCs: 1) mosaic analysis of *thread* (or DIAP1) null GSC and CySC clones, and 2) global RNAi-mediated knockdown of DIAP1. In the first approach, we used two loss-of-function alleles of *thread* to create negatively marked DIAP1 null CySC and GSC clones (see Methods). By tracking clones through the absence of GFP, we found that by 2 days after clone induction (ACI) there were already significantly fewer DIAP1 null GSCs and CySCs compared to control wild-type clones (Fig. 2.3A-C, Table 2.1). This suggested that the majority of DIAP1 null stem cells are lost rapidly, since most were no longer visible by 2 days ACI, which is the earliest time point the marking system is visible (Corish and Tyler-Smith, 1999). The loss of DIAP1 null stem cells could be due to their differentiation or death. If stem cell clones are lost by differentiation we would expect to see a robust number of differentiating clones 2 days ACI. Compared to wild type clones, very few differentiating germline and somatic cells lacking DIAP1 were detected (Table 2.1), suggesting that the DIAP1 null GSC and CySC clones are rapidly dying, rather than entering the differentiation pathway. Consistent with this observation, DIAP1 null

clones are lost very rapidly in the larval imaginal disc, the adult eye, and the ovary due to cell death (Hay et al., 1995; Ryoo et al., 2004), and DIAP1 ablation in spermatogonial cysts results in their death (Yacobi-Sharon et al., 2013). Therefore, we consider it most likely that DIAP1 null clones in the adult testis are rapidly lost due to cell death, rather than differentiation. We did notice that DIAP1 null GSCs were lost less rapidly than the DIAP1 null CySCs (Table 1), which suggests that they might contain other anti-apoptotic proteins apart from DIAP1. This possibility is consistent with the presence of alternative germ cell death pathways in spermatogonia (Yacobi-Sharon et al., 2013).

To confirm and extend our clonal analysis, we performed conditional global induction of DIAP1 RNAi to reduce DIAP1 levels in adult testes. Induction of DIAP1 RNAi (under control of the Heat shock promoter) resulted in a reduction of DIAP1 protein levels throughout the entire testis including the niche region (Fig. S2.2A-D). Prior to RNAi induction, very few dying cells were detected in the stem cell zone in both control and experimental testes. At 3 hours post RNAi induction, the number of TUNEL-positive cells within the stem cell zone increased significantly in testes lacking DIAP1 relative to controls (from 0.18 in un-induced DIAP1 RNAi control to 1.36 dying cells per testis, Fig. 2.3D-F) (the few dying stem cells in un-induced DIAP1 RNAi control could be due to background induction of DIAP1 RNAi at 25 ^{o}C). Importantly, this increase in TUNEL-positive cells is not due to experimental conditions: testes expressing a control RNAi (against GFP) did not have a significant increase in number of TUNEL-positive cells upon heat-shock (Fig. 2.3F). Finally, expressing DIAP1 RNAi specifically in the CySC lineage significantly decreased the

number of CySCs (Fig. S2.3A). The significant increase in stem cell death upon DIAP1 RNAi induction, coupled with the rapid loss of DIAP1 null stem cell clones, support the conclusion that DIAP1 is required for survival of GSCs and CySCs in the testis niche.

Decreased JAK-STAT signaling makes the stem cells susceptible to stress-induced death

Reducing Stat levels (by shifting stat temperature-sensitive flies to the non-permissive temperature) causes a decrease in DIAP1 levels (Fig. 2.2), but stem cells in these testes undergo differentiation, rather than cell death (Brawley and Matunis, 2004), suggesting that the low levels of DIAP1 that remain after reduction of STAT are sufficient to protect stem cells from death under physiological conditions. However, it also suggested that the reduced levels of DIAP1 would render these cells more sensitive to stress-induced cell death. To test this hypothesis, we irradiated flies with decreased STAT levels (see Methods) with 25 Gy IR and then used TUNEL staining to quantify the number of dying cells in the testis apex. We found significantly more TUNEL positive cells in the stem cell zone in the irradiated testes with decreased JAK-STAT signaling (4.11 dying cells per testis apex, N=27 testes) compared to irradiated controls with normal JAK-STAT signaling (0.8 dying cells per testis apex, N=30 testes; Fig. 2.3G-I). We conclude that decreasing JAK-STAT signaling sensitizes the stem cells to stress-induced cell death, likely because of reduction in DIAP1 levels.

Up-regulation of DIAP1 in differentiating somatic cells, but not germ cells, rescues stress-induced spermatogonial death

Stem cells in the Drosophila testis resist cell death better than their differentiating progeny (Fig. 2.1), have an enrichment of DIAP1 (Fig. 2.2), and require this anti-apoptotic protein for their survival (Fig 2.3). However, since dying stem cells are never detected in flies kept under standard laboratory conditions, we cannot tell if the higher levels of DIAP1 within stem cells are sufficient to confer their survival advantages. Ideally we could reduce the DIAP1 levels in the stem cells to match those seen in spermatogonia, and then ask if stem cells die more often, but this is not technically possible: even moderate RNAi knockdown of DIAP1 causes stem cells to die, and flies heterozygous for the null allele *thread*^{4} did not show a noticeable increase in stem cell death in the testis (data not shown). Similarly, *thread* null heterozygotes do not show any increase in neuronal or eye cell death (Choi et al., 2006; Hawkins et al., 2000; Wang et al., 1999). Thus, though indirect reduction of DIAP1 by decreasing JAK-STAT signaling rendered the stem cells susceptible to stress-induced death, the precise level of DIAP1 required for stem cell maintenance in the testis is not known. We do know, however, that ectopically up-regulating DIAP1 levels in GSCs or CySCs does not adversely affect their viability: we never saw dying GSCs or CySCs in testes where DIAP1 is overexpressed in GSCs or CySCs respectively (Fig. 2.4A-B' and 2.4D, D') (data not shown). Furthermore, the Arama lab showed that up-regulating DIAP1 in differentiating germ cells is not sufficient to rescue these cells from death, a result that we have confirmed using both Nanos and Bam-Gal4 drivers (Fig. 2.4E, Fig. S2.4B-D). This is because, as mentioned above,

normal spermatogonial death occurs by a combination of apoptosis and necrotic pathways and does not involve the canonical apoptotic pathway, which DIAP1 inhibits (reviewed in Fuchs and Steller, 2011; Yacobi-Sharon et al., 2013). Therefore, we wondered if stress-induced cell death could differ from the non-canonical cell death that spermatogonia undergo under normal laboratory conditions (Yacobi-Sharon et al., 2013). If so, stress-induced cell death could be rescued by up-regulation of DIAP1 in the spermatogonia. To explore this possibility, we up-regulated DIAP1 in 4-16 cell spermatogonial clusters (using the Bam-Gal4 driver) and subjected the flies to stress (protein starvation and irradiation). We found that the spermatogonial cell death induced by protein starvation or irradiation is not rescued by up-regulation of DIAP1 (Fig. S2.4A-C). We conclude that up-regulating DIAP1 in the late spermatogonial cells is insufficient to autonomously rescue them from cell death that occurs under laboratory conditions, or under starvation-induced stress.

Since over-expressing DIAP1 directly in the germ cells was not sufficient to promote their survival under protein starvation conditions, we hypothesized that some spermatogonia may be dying as a secondary consequence of the death of their accompanying somatic cyst cells. Cyst cells envelop differentiating germ cells (reviewed in Zoller and Schulz, 2012) and are vital for the survival and proper differentiation of the spermatogonia (Fig. S2.3B)(Hetie et al., 2014; Lim and Fuller, 2012; reviewed in Zoller and Schulz, 2012). Up-regulating DIAP1 levels in cyst cells using the c587 Gal4 driver (Fig. 2.4D, D') in flies reared under normal conditions had no effect on spermatogonial cell death, indicating that these germ cells are not likely dying due to cyst cell death (Fig 2.4F, G controls). However, the additional dying

spermatogonia that arise after protein starvation or irradiation are rescued by boosting DIAP1 levels in the CySC lineage: the average number of dying spermatogonial clusters in starved males decreased significantly from 2.92 to 1.97 upon overexpression of DIAP1 in cyst lineage cells (N= 24, 44) ($p<0.05$) (Fig. 2.4F). Similarly, DIAP1 over-expression in the CySC lineage rescued the additional spermatogonial death induced by irradiation. The average number of dying spermatogonial clusters per testis apex in flies subjected to 25 Gy of γ-radiation (which kills spermatogonia and somatic cyst cells but not stem cells) (Fig. 2.1) decreased significantly from 1.34 to 0.69 upon overexpression of DIAP1 in CySC lineage cells ($p<0.01$) (Fig. 2.4G). This is similar to the level of death seen in un-irradiated control flies without DIAP1 up-regulation (Fig. 2.4F, G). We conclude that expression of ectopic DIAP1 in the somatic cells non-autonomously rescues stress-induced spermatogonial cell death.

If overexpression of DIAP1 in CySC lineage cells but not in germ cells is sufficient to rescue stress-induced spermatogonial cell death, then overexpression of DIAP1 in both somatic and germ cells should pheno-copy over-expression of DIAP1 in the CySC lineage alone. Therefore, we used a tubulin Gal4 driver to overexpress DIAP1 in both the somatic and germline cells (reviewed in White-Cooper, 2012). Consistent with published results, and our data in Fig. 2.4E-G, we did not observe any rescue in spermatogonial death in these flies prior to irradiation (Yacobi-Sharon et al., 2013)(Fig. 2.4H). After 25 Gy of irradiation, we found a significant decrease in the average number of dying spermatogonial clusters (from 4.2 to 2.3 per testis apex) ($p<0.01$) upon overexpression of DIAP1 in germline and soma. Thus, ectopic DIAP1

rescues close to 50% of the spermatogonial clusters from IR induced death when it is expressed in either the germline and soma or the soma alone. Taken together, these results indicate that stress-mediated increase of spermatogonial cell death can be completely rescued non-autonomously by over-expression of DIAP1 in the somatic CySC lineage.

Cyst cells are more susceptible than germline cells to stress-induced cell death

Since we could fully rescue the stress-induced increase in spermatogonial cell death by over-expressing DIAP1 in somatic cyst cells but not in germ cells, we hypothesized that this increase in spermatogonial cell death may be secondary to the loss of the cyst cells. If so, we may be able to detect an increase in the number of dying cyst cells adjacent to living germ cell clusters under stress conditions. To identify dying cyst cell-living germ cell cluster pairs, we used the antibody against cleaved Dcp-1 protein, which marks dying somatic cells under normal and stress conditions (Fig. S2.1A, B), an antibody against Traffic jam to mark somatic cell nuclei, and expression of Bam-GFP fusion protein to mark spermatogonial cell clusters. We assumed that the germline cells which are expressing Bam-GFP are still alive. We counted the number of dying cyst cell-living germ cell pairs in testes from both irradiated (25 Gy) and un-irradiated controls. The number of these pairs increased significantly from an average of 0.6 to 1.1 per testis apex upon irradiation (N=20 testes) (Fig 2.5A-B). We hypothesize that the Dcp-1 positive cyst cells in these pairs are expressing activated effector caspase and are progressing through apoptosis while the enclosed germline cells are living. However, we cannot rule out the possibility that the enclosed germ cells are expressing cell death markers different

from cleaved Dcp-1, since markers of early stages of germ cell death that can be visualized along with cell-specific protein expression have not yet been identified. Our data therefore suggest that cyst cells are more susceptible to stress-induced cell death than the enclosed germline cluster and start progressing towards cell death when the associated germline cluster is still living.

Altogether, our results support a model where niche signaling makes the stem cells resistant to stress-induced cell death. The differentiating cells lack the resistance to stress-induced cell death, with cyst cells being more susceptible to stress-induced cell death than their accompanying germ cell partners.

Discussion

Both germline and somatic stem cells in the adult Drosophila testis are more resistant to apoptosis-inducing stimuli than their differentiating daughters. Niche signaling causes enhanced expression of DIAP1 in both GSCs and CySCs, and the resistance to apoptosis is mediated, at least in part, by this enhanced expression.

Niche signaling can enhance survival of stem cells

Stem cells in various mammalian adult tissues have enhanced expression of anti-apoptotic proteins such as Bcl2 and IAPs. However, little is known about how these proteins are regulated, or whether signals from the niche play a role in their up-regulation. Here, we find that niche signaling can play a direct role in promoting survival of stem cells by up-regulating the expression of pro-survival genes. We show that the anti-apoptotic protein DIAP1 is a transcriptional target of JAK-STAT signaling in both the CySCs and the GSCs in the testis. STAT binds directly to the DIAP1 promoter region, and this binding is sufficient to cause up-regulation of DIAP1 in 3rd instar larval wing discs (Betz et al., 2008a). Therefore, it is likely that STAT acts directly on DIAP1 in testis stem cells, but future experiments are needed to determine this. STAT has also been shown to up-regulate anti-apoptotic proteins in cycling adult satellite stem cells in mammalian muscle (Golding et al., 2007), but whether STAT activation is a consequence of niche signaling in this tissue remains to be determined.

Since DIAP1 is a STAT target, and loss of DIAP1 leads to cell death, one might expect STAT null testes to lose stem cells by cell death. However, STAT null testes appear to lose stem cells by differentiation and not cell death (Brawley and

Matunis, 2004). This could be due to the fact that STAT is a transcription factor regulating the expression pattern of multiple genes. Some of these genes regulate stem cell renewal, and it is possible that reduced expression of these genes causes stem cell differentiation to occur before DIAP1 expression is reduced enough to cause cell death. Alternatively, DIAP1 may also be regulated by another pathway in the testis and its levels may not be affected at the same rate as some of the more direct targets of STAT (such as the targets involved in renewal). Consistent with this idea, DIAP1 levels are not reduced completely upon reduction of STAT (see methods for STAT reduction procedures). Although the decrease in STAT levels does not induce cell death in the stem cells, similar decrease makes them sensitive to stress-induced cell death. Identifying other regulators of DIAP1, which may be involved in DIAP1 up-regulation in stem cells, could help to distinguish between these possibilities. Other targets of niche signaling may also have an anti-apoptotic roles in stem cells. Possible candidates include the IAP family member Drosophila inhibitor of apoptosis 2 (DIAP2). According to published RNA-seq data there seems to be an enrichment of DIAP2 in testes (Chintapalli et al., 2007). However, although DIAP2 is an IAP family member, so far it has been thought to be primarily important for innate immunity, rather than cell survival (Gesellchen et al., 2005; Huh et al., 2007; Leulier et al., 2006).

Cyst cells play a role in germ cell survival

Cyst cells are known to play a role in germ cell survival (Hetie et al., 2014; reviewed in Zoller and Schulz, 2012). Our data shows that cyst cells can be responsible for death of germ cell clusters due to their susceptibility to stress-induced cell death. The

susceptibility of cyst cells to stress conditions could be one of the reasons why stress-induced spermatogonial cell death can be rescued non-autonomously by DIAP1 overexpression in cyst cells. Similar non-autonomous regulation of germline cell death by somatic cell has been demonstrated in adult Drosophila ovary where induction of cell death in follicle cells (somatic lineage) lead to nurse cell death (germ cell lineage) (Chao and Nagoshi, 1999). It may be interesting to analyze why cyst cells are more susceptible to stress-induced death than their enclosed germ cells as well as what kind of cross-talk goes between the cyst cells and their enclosed germ cell clusters that leads to germ cell death once cyst cells are lost. Our data, therefore, supports and extends the published work further, providing another role of cyst cells in survival of the enclosed germ cell cluster. However, we must note that, although DIAP1 over expression in cyst cells can prevent stress-induced spermatogonial cell death, it does not rescue normal spermatogonial death.

GSCs in the testis are radio-resistant

The enhanced capability of male GSCs to survive radiation-induced cell death has been well documented in Drosophila testis (Welshons and Russel, 1957). Classic studies in Drosophila showed that following irradiation, males are fertile for a while then enter a period of temporary sterility, before recovering fertility (Welshons and Russel, 1957). The period of temporary sterility is proportional to the intensity of radiation (Welshons and Russel, 1957). Welshons and Russel studied the morphological characteristics of the testis after irradiation and concluded that the testis looked completely normal 15 days post-irradiation with a 4000 roentgen (35 Gy) X ray dosage (Welshons and Russel, 1957). Microscopic observation of the

irradiated testes indicated that the spermatogonia are susceptible to radiation and undergo apoptosis while the spermatocytes were relatively more resistant to radiation (Welshons and Russel, 1957). Although the fate of the stem cells in these studies was not determined, the full restoration of fertility after a temporary lapse suggests that the stem cells were resistant to radiation. Our work analyzes the effect of irradiation on stem cells and explains the phenomenon of temporary sterility. The stem cells are not as susceptible as the spermatogonia to radiation-induced apoptosis, and survive, providing a source of new germ cells post-irradiation. Thus, our work indicate that temporary sterility is induced due to the elimination of spermatogonia upon irradiation, and fertility is restored when the stem cells replenish the spermatogonial pool. The ability of male GSCs to resist death extends to mammalian systems including rat and mouse testis, where GSCs can also divide to restore the fertility of irradiated mice and rats (Dym and Clermont, 1970; Erickson, 1976; Withers et al., 1974). Our data, agrees with the mammalian data and suggests that niche signals may make GSCs more resistant to stress conditions than their differentiating progeny across a diverse set of organisms.

Implications in regulation of cancer stem cells

Up-regulation of anti-apoptotic proteins in stem cells is especially important in diseases such as cancer. Many cancers such as leukemia, glioblastoma, and breast and skin cancers are known to arise from cancer stem cells, which are capable of forming new tumors (reviewed in Blanpain et al., 2011b; Clarke and Fuller, 2006; Reya et al., 2001). Cancer stem cells have been shown to be resistant to both radiation and chemical agent-induced apoptosis (reviewed in Abdullah and Chow, 2013; Pajonk et

al., 2010). This resistance reduces the efficacy of cancer treatments because even when the bulk of the tumor is reduced, the cancer stem cells survive and often reform the tumor (reviewed in Yu et al., 2012). One suspected reason for apoptosis resistance in cancer stem cells could be enhanced expression of anti-apoptotic proteins (reviewed in Wicha et al., 2006). Indeed, a gene expression analysis of patient-derived glioblastoma stem cells (which are $CD133^{+}$) found enhanced expression of anti-apoptotic genes such as Bcl-2, XIAP and Survivin among others, compared to the $CD133^{-}$ cells which do not form tumors (Liu et al., 2006). Similar to normal stem cells, cancer stem cells are also affected by signals from niche (reviewed in Plaks et al., 2015; Schepers et al., 2015, 2013). It is possible that niche signals are responsible for this increased expression of anti-apoptotic genes in cancer stem cells; therefore, niche cells may be a better therapeutic target than cancer cells (which includes the cancer stem cells) for cancer treatment.

In conclusion our results in this study not only demonstrate the significance of niche signals in stem cell survival in normal as well as stress conditions, but also underline a non-autonomous manner in which somatic support cells can lead to the death of adjacent germ cell cluster.

Materials and Methods

Fly stocks and cultures

Flies were raised on standard yeast medium at 25˚C unless otherwise noted. The following stocks were used: *Bam-GFP* (kind gift of Dr. D. McKearin)(Chen and McKearin, 2003), *UASp-DIAP1* (kind gift of Dr. K. McCall) (Peterson and McCall, 2013), *Nanos-Gal4* (kind gift of Dr. E Selva) (White-Cooper, 2012), *Bam-Gal4* (kind gift of Dr. D. McKearin)(White-Cooper, 2012), *c587-Gal4* (kind gift of Dr. A. Spradling)(Skora and Spradling, 2010), *HS-Upd* (kind gift of Dr. D. Harrison) (McGregor et al., 2002), *UASt-DIAP1-RNAi* (kind gift of Dr. A. Bashirullah) (Huh et al., 2004) and *HS-DIAP1-RNAi* (kind gift of Dr. A. Bashirullah) (Yin and Thummel, 2004). Other stocks were from the Bloomington Drosophila Stock Center (BDSC).

Starvation

Flies were transferred to standard vials containing apple juice-agar food (Etchegaray et al., 2012) without yeast for a period of 16-24 hours. Control flies were kept in identical conditions on standard yeast medium.

DIAP1 RNAi induction

Using Heat Shock

0-4 day old *HS-DIAP1-RNAi* male flies were heat-shocked in a 37˚C water bath for 1 hour, returned to 25˚C for 3.5 hours, and then dissected.

Using CySC and Cyst cell driver

C587 Gal4;; tub-Gal80^{ts} female flies were crossed with male *;UAS DIAP1 RNAi;* flies and maintained at 25 ºC. 0-4 day old male progeny were collected from the cross and kept at 31 ºC for 6 days and then dissected and stained with TUNEL to look at dying spermatogonia.

C587 Gal4;; tub-Gal80^{ts} female flies were crossed with male *;UAS DIAP1 RNAi/SM6b;UAS Dicer2* flies and maintained at 18 ºC. 0-4 day old male progeny were collected from the cross and kept at 31 ºC for 14 days and then dissected. Zfh-1 staining was used to count CySCs. *C587 Gal4; SM6b; tub-Gal80^{ts}/UAS Dicer2* flies were used as sibling controls.

Mosaic analysis

The FLP-mediated mitotic recombination technique (Xu and Rubin, 1993) was used to generate negatively marked *thread* homozygous mutant GSC and CySC clones. Newly eclosed males of the genotype *Hs-FLP/Y; FRT2A th^5 / FRT2A Ubi-GFP::nls* or *Hs-FLP/Y; FRT2A th^4 / FRT2A Ubi-GFP::nls* (experimental) or *Hs-FLP/Y; FRT2A/FRT2A Ubi-GFP::nls* (control) were heat-shocked as described in (Issigonis and Matunis, 2012) and then dissected 2, 4, 6, 8 or 10 days after clone induction. GSC clones were identified by the absence of GFP and absence of the somatic markers Zfh1 or Traffic jam (Tj) as well as by their position adjacent to the hub. CySC clones were identified by the absence of GFP, presence of Zfh1 or Tj, and position within 2 cell diameters of the hub. Statistical analysis on the percentage of testes with clones (normalized to basal clone induction rates obtained from non-heat-shocked controls) was performed using the Fisher Exact or Chi-Squared tests.

HS-Upd *induction and* Stat92E^{F} *temperature shift*

To reduce JAK-STAT signaling, temperature sensitive *Stat92E^{F}/Stat92E^{06346}* and control *Stat92E^{F}/TM3* flies were raised at 18˚C; 0-4 day old adult male flies were placed at 29 ˚C for 24 hours and then dissected. For irradiation after STAT reduction, temperature sensitive *Stat92E^{F}/Stat92E^{06346}* and control *Stat92E^{F}/TM3* flies were irradiated immediately after 24 hours at 29 ˚C, then placed at 25 ˚C and dissected 3 hours after irradiation. To increase JAK-STAT signaling, *HS-Upd/Y* and control *FM7/Y* adult male flies were heat shocked in a 37˚C water bath for 45 minutes, returned to 25˚C for 30 minutes, and then dissected.

γ-irradiation

0-4 day old adult male flies were kept in a vial with standard yeast medium and exposed to a 25Gy or 50Gy dose of γ-radiation in a Gammacell 3000 Elan with Cesium 137 as the radiation source. The testes were dissected out, fixed and immunostained 3 hours post irradiation.

Testis immunocytochemistry

Testes were dissected, fixed, and immunostained as described in (Matunis et al., 1997). The following primary antibodies were used: rabbit anti-Vasa at 1:200 (Santa Cruz Biotechnology); mouse anti-DIAP1 at 1:100 (kind gift of Dr. B. Hay); rabbit anti-cleaved Dcp-1 at 1:100 (Cell Signaling); guinea pig anti-Zfh-1 at 1:500; chicken anti-GFP at 1:10,000 (Abcam); and guinea pig anti-Traffic Jam at 1:10,000 (kind gift of Dr. D. Godt). Alexa fluor-conjugated secondary IgG (H+L) antibodies (Molecular

Probes/Invitrogen) were used at 1:200. Nuclei were counterstained with 1 μg/ml 4′-6-diamidino-2-phenylindole (DAPI) (Roche Molecular Biochemical). Stained testes were mounted and imaged in Vectashield (Vector Labs).

Image analysis for fluorescence quantification

Confocal images were acquired in the linear range for intensity for all channels on a Zeiss LSM 5 PASCAL microscope or on a Zeiss LSM 700 microscope. ImageJ (Schneider et al., 2012) was used to identify the optical section of each testis where the hub had the largest diameter and to measure the DIAP1 fluorescence intensity in GSCs and CySCs in that section. GSCs were identified as Zfh1-negative cells next to hub, and CySCs were identified as Zfh1-positive cells within two cell diameters of the hub. Since the hub has very low amounts of DIAP1, we used the fluorescence intensity of the hub as background and subtracted it from the intensity measurement in each stem cell. We then normalized the intensity measurement for each stem cell by comparing it to the DAPI fluorescence intensity for the same cell using a similar approach as published (Nguyen et al., 2015; Starz-Gaiano et al., 2008)

Cell death analysis

Specification of the zones: This analysis was performed using flies expressing the fusion protein Bam-GFP. The testis apex was divided into a stem cell zone and a differentiating cell zone. The stem cell zone was defined as all cells within the first two tiers of nuclei surrounding the hub, and includes GSCs and CySCs and some of their immediate daughters. The differentiating cell zone was defined as all cells

outside the stem cell zone and up to the posterior end of the Bam-positive zone, and includes mostly 2, 4, and 8-cell spermatogonial cysts.

Detection of apoptosis: To detect apoptotic cells, we used terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Testes were fixed for 20 minutes in 4% formaldehyde in PBX (Matunis et al., 1997), washed 2 x 10 minutes in PBX, and labeled according to the manufacturer's instructions with the Millipore Apoptag kit S7165.

Quantification: To quantify apoptotic cells, we counted TUNEL- positive spots in the stem cell and differentiating cell zones. To quantify dying spermtogonia, we counted only those TUNEL-positive spots in the differentiating cell zone that had a diameter ≥ 5 μm.

Statistical Analysis

All statistical tests were performed using Prism 6 (GraphPad Software).

Author Contributions

S.H., P.H. and E.M. designed the experiments. S.H. and P.H. performed experiments and data analysis. S.H. and E.M. wrote the manuscript.

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Table Legend

Table 2.1. DIAP1 is required for maintenance of both GSCs and CySCs.

Testes with one or more clones have been quantified both as a fraction of total number of testes and as percentages (in brackets). P-values based on Chi Square and Fisher's exact test. p-values are based on comparisons between the percent testes with wild-type GSC or CySC clones and percent testes with DIAP1 null GSC or CySC clones (including both *thread*^{4} and *thread*^{5} clones*)*. HS= Heat shock, ACI= After clone induction.

Figure Legends

Figure 2.1. Stem cells in the testis niche are more resistant to radiation and starvation induced cell death than differentiating cells.

(A) Illustration of the Drosophila adult testis niche. GSCs (yellow) and CySCs (dark blue) are adjacent to the hub at the testis apex. GSCs divide to give rise to gonialblasts, which continue to divide, forming spermatogonial cysts. CySCs divide to form cyst cells, which envelop gonialblasts and their progeny. TUNEL-positive cells were quantified in two adjacent regions of the testis 1), A "Stem cell zone" comprised of the first two tiers of cells adjacent to the hub which includes all stem cells (GSCs and CySCs) and an occasional gonialblast and/or early cyst cell. 2), A "Differentiating cell zone", which extends from the boundary of the stem cell zone through the Bam-GFP positive spermatogonial cysts (green).

(B, C, D) Confocal sections of testes expressing Bam-GFP fusion protein and immuno-stained with anti-GFP antisera (Bam expression pattern- spermatogonial cysts, green), TUNEL (apoptotic cells, red) and DAPI (DNA, blue) under control conditions (B), after 3 hours of exposure to 25 Gy γ radiation (C) and after 19.5 hours of protein-starvation (D). TUNEL-positive spots (arrows) are typically seen within and adjacent to Bam-positive spermatogonial cysts (C, D). Scale bar 10 μm.

(E, F) Scatter plots showing the number of TUNEL-positive spots per testis apex in the stem cell and differentiating cell zones. (E) Upon 25Gy of irradiation, there is no significant difference in TUNEL-positive spots in stem cell zone compared to un-irradiated controls. In contrast, 50 Gy of IR causes significantly more stem cells to

die. In the differentiating cell zone, significantly more cells die under both 25Gy and 50Gy doses compared to un-irradiated controls. (*, $p<0.05$), (**, $p<0.01$). 2-tailed Kruskal-Wallis test with multiple comparisons. Bars indicate mean and S.D.

(F) There is no significant difference in TUNEL-positive spots in the stem cell zone after starvation. There is a significant increase in TUNEL-positive spots in differentiating cell zone after starvation. (*, $p<0.05$). 2-tailed Mann Whitney test.

Figure 2.2. DIAP1 is enriched in the stem cells and is a target of JAK-STAT signaling.

(A-C) Confocal sections through the apex of testes stained with anti-DIAP1 (white), anti-Zfh1 (CySCs and cyst cells, red), and DAPI (DNA, blue). (A'-C') DIAP1 channel alone. In control testes, DIAP1 protein is enriched around the hub (white outline) and co-localizes with CySCs (Zfh1 positive cells near the hub; one indicated, arrowhead) and GSCs (Zfh1- negative cells contacting the hub; one indicated, arrow).

(B) DIAP1 levels are reduced in testes with reduced JAK-STAT signaling (genotype: $stat^{F}/stat^{06436}$) after 24h at restrictive temperature (see methods).

(C) DIAP1 levels are increased in both stem cell populations and in differentiating cells in testes with increased JAK-STAT signaling (*hs-upd* testes after 45 minutes of induction). (D, E) Bar graph showing quantification of DIAP1 levels in stem cells normalized to DAPI levels in the same cells in control (green) and experimental (red) flies when JAK-STAT is reduced (D) or up-regulated (E).(***, $p<0.001$), (****, $p<0.0001$). 2-tailed Mann Whitney test. Error Bars indicate mean and S.E.M.

Figure 2.3. DIAP1 is required for stem cell survival.

(A, B) Confocal sections through the testis apex show control (A) or DIAP1 null (*thread*^{5}, B) clones 2 days after clone induction (ACI). Hubs are outlined in white (thick line). Negatively marked clones, identified by the absence of GFP (green) staining, are outlined in white (thin line). Clones positive for Tj (red) are CySC lineage clones (arrowheads), and those negative for Tj are germ line clones (arrows). Both control (A, A') and DIAP1 null (B, B') clones can be seen next to the hub. (C) Line diagram showing the percentage of testes with DIAP1 null (*thread*^{5}, Exptl) or control (Cntrl) GSC or CySC clones after clone induction. Very few *thread*^{5} CySC clones (blue solid line) and *thread*^{5} GSC clones (red solid line), are seen compared to control CySC (blue dashed line) or GSC (red dashed line) clones. (D-H) Confocal sections through the apex of testes stained with TUNEL (red) and DAPI (blue). Hubs are outlined in white. (D'-H'), TUNEL channel alone. (D, D') TUNEL positive cells next to the hub were rarely seen in control testes after induction of GFP RNAi. (E, E') After heat shock-mediated induction of DIAP1-RNAi (E), TUNEL positive cells were seen adjacent to the hub (arrow in E, E'). (F) Scatter plot showing quantification of data from D and E. The number of dying cells in the stem cell zone (see methods) increased significantly upon induction of DIAP1 RNAi compared to induction of GFP RNAi in controls (*, $p<0.05$). No significant difference in cell death was observed without induction of RNAi or with induction of GFP RNAi. Statitistical test used is Kruskal – Wallis test of multiple comparisons. (G, G') Irradiation of control testes (genotype- *stat*^{F} */ TM3*, after 24h at restrictive temperature*)* at 25 Gy did not result in TUNEL positive cells next to the hub (stem cell zone). (H, H') Irradiation of

testes with reduced JAK-STAT signaling (genotype: $stat^{F}/stat^{06436}$, after 24 h at restrictive temperature) at 25 Gy resulted in increased number of TUNEL positive cells next to the hub (arrows in G, G') (stem cell zone). Scale bars 10 μm. (I) Scatter plot shows quantification of data from G and H. The number of TUNEL positive cells in the stem cell zone increased significantly upon irradiation (25 Gy) after reduction of JAK-STAT signaling compared to irradiated control testes (****, $p<0.0001$). 2 tailed Mann-Whitney test. Bars (F, I) indicate mean and S.D.

Figure 2.4. **DIAP1 over-expression in CySCs and their daughters rescues differentiating germline cells from stress induced cell death.**

(A -D) Confocal sections through the apex of testes stained with anti-DIAP1 (white), anti-Vasa (A-C, germ cells, red) or anti-Tj (D, somatic cells, red), and DAPI. (A'-D') DIAP1 channel alone. Testes were imaged at the same gain to show different levels of DIAP1 protein in (A, A') a control testis or in testes overexpressing DIAP1 in (B, B') GSCs and their daughters using Nanos Gal4; (C, C') in both germ cells and somatic cyst cells using Tubulin Gal4; (D, D') in CySCs and their daughters using c587 Gal4. Hubs are outlined in white. Scale bar, 10 μm. (E-H) Scatter plots showing quantification of TUNEL-positive spermatogonial cell clusters (see text) under normal conditions or under stress from protein starvation (E-F) or irradiation (G-H). Over expression of DIAP1 in GSCs and their daughters (E) under normal or protein starvation conditions does not significantly alter the number of TUNEL positive spermatogonial cell clusters compared to controls without DIAP1 over expression. However, there is a decrease in TUNEL positive spermatogonial cell clusters under both protein starvation and irradiation conditions in testes over expressing DIAP1 in

CySCs and their daughters (F, G). Simultaneous over expression of DIAP1 in both germ cells and somatic cyst cells also results in a decrease in TUNEL positive spermatogonial cell clusters under irradiation conditions (H).

(*, $p<0.05$), (**, $p<0.01$). 2-tailed Mann-Whitney test. In all graphs, bars indicate mean and S.D.; sample size for each treatment set indicated under the respective plot.

Figure 2.5. Cyst cells die before the enclosed germ cell clusters under stress condition.

(A –A") Confocal section through the apex of a testis expressing Bam-GFP fusion protein and stained with anti-TJ (somatic cells, white, A'), anti- cleaved Dcp-1 (Death caspase-1: effector caspase activity, red, A") or anti-GFP (Bam expression pattern: germ cell clusters, green), and DAPI. (A) A dying cyst cells (TJ positive) with cleaved Dcp-1 (white arrow) was seen adjacent to a living (cleaved Dcp-1 negative) germ cell cluster (Bam expression) after irradiation (25 Gy). Scale bar 10 μm. (B) Scatter plots showing quantification of dying cyst cell (TJ positive, cleaved Dcp-1 positive)-living germ cell cluster (Bam expression) pair in a spermatogonial cyst under control or irradiation conditions. Dying cyst cell-living germ cell cluster pairs are seen in control as well as irradiated testes. Significantly more dying cyst cell-living germ cell cluster pairs are seen under irradiation conditions (*, $p<0.05$). 1-tailed Student's T-test. In all graphs, bars indicate mean and S.D., sample size for each treatment set indicated under the respective plot.

Supplementary Figure Legends

Figure S2.1. Cleaved Dcp-1 is expressed in control as well as irradiated testes.

(A-B') Confocal sections of testes expressing Bam-GFP fusion protein and immuno-stained with anti-GFP antisera (Bam expression pattern- spermatogonial cysts, green), anti- cleaved Dcp-1 (Death caspase activity, red), anti- TJ (white, somatic cells) and DAPI (DNA, blue) under control conditions (A,A') and after 3 hours of exposure to 25 Gy IR (B,B'). (A'-B') TJ channel alone. Cleaved Dcp-1 positive cyst cells were seen in both (A) and (B) (arrow in (A, B) and (A', B')). More cleaved Dcp-1 positive cells were seen in (B) than in (A). Hub is not visible in the confocal plane in (B, B'), its position is marked with white asterisk. Hub is outlined in white in (A, A'). Scale bar 10 μm.

(C) Scatter plots showing the number of cleaved Dcp-1 positive somatic cells per testis apex in the stem cell zone and the differentiating cell zone. Upon 25Gy of irradiation, there is no significant difference in cleaved Dcp-1 positive CySCs in stem cell zone (2-tailed Mann-Whitney test) but there is a significant increase in cleaved Dcp-1 positive cyst cells in differentiating cell zone (*, $p<0.05$). 2-tailed T-test. Bars indicate mean and S.D.

Figure S2.2. Induction of DIAP1 RNAi results in loss of DIAP1.

(A-D') Confocal sections through the apex of testes stained with anti-DIAP1 (white), anti-Vasa (germ cells, red) and DAPI (blue). (A'-D') DIAP1 channel alone. Hubs are outlined in white. Testes were scanned at same gain. (B) DIAP1 levels are reduced in *hs-DIAP1-RNAi* testes after heat shock-mediated induction of DIAP1-RNAi. (A)

Wild-type expression levels of DIAP1 are observed in *hs-DIAP1-RNAi* testes without heat shock as well as in *hs-Gal4>UAS-GFP-RNAi* testes before or after heat shock-mediated induction of GFP RNAi (C, D). Scale bar 10 μm.

Figure S2.3. Somatic induction of DIAP1 RNAi results in loss of CySCs and spermatogonial clusters.

(A, B) Scatter plots showing quantification of CySCs (A) and TUNEL positive germ cell clusters (B) in control testes and in testes where DIAP1 was knocked down specifically in CySCs and cyst cells (using c587 Gal4) by incubating the flies at 31 ^{0}C for 14 days (A) or 6 days (B). The temperature sensitive Gal80 protein was used to overcome embryonic lethality in both (A) and (B). Dicer2 was also used in (A) to increase the effectiveness of DIAP1 knockdown (see Methods). (A) Somatic ablation of DIAP1 resulted in significant decrease in the number of CySCs from 12.8 CySCs to 6.73 CySCs per testis apex. (***, $p<0.001$). 2-tailed T-test. (B) Dying spermatogonial clusters were identified as TUNEL spots that were 5 μm or more in diameter (see Methods). We found a significant increase in spermatogonial cell death from 1.07 to 1.85 dying spermatogonial clusters per testis apex upon DIAP1 knockdown in CySCs and cyst cells. (*, $p<0.05$). 1-tailed Mann-Whitney test.

Figure S2.4. DIAP1 overexpression in germ cell does not rescue stress induced cell death.

(A –A') Confocal section through the apex of testes stained with anti-DIAP1 (white), anti-Vasa (A-C, germ cells, red) and DAPI. (A') DIAP1 channel alone. (A, A') DIAP1 over expressed specifically in spermatogonial clusters using Bam Gal4. Hub

is outlined in white. Scale bar, 10 μm. (B-D) Scatter plots showing quantification of TUNEL-positive spermatogonial cell clusters (see text) under normal conditions or under stress from protein starvation (B) or irradiation (C, D). Over expression of DIAP1 in spermatogonial clusters using Bam-Gal4 under (B) protein starvation or (C) irradiation conditions does not significantly alter the number of TUNEL positive spermatogonial cell clusters compared to controls without DIAP1 over expression. Similarly, there is no change in the number of TUNEL positive spermatogonial cell clusters under irradiation conditions in testes over expressing DIAP1 in GSCs and their immediate daughters using Nanos Gal4 (D). 2-tailed Mann-Whitney test is used as statistical test in all plots. In all graphs, bars indicate mean and S.D., sample size for each treatment set indicated under the respective plot.

Table

Table 2.1

Days ACI	*thread* allele	Percentage of testes with clones			
		GSC clones	CySC clones	Spermatogonial Clones	Cyst Cell Clones
No HS control	Wild type	6.3% (6/95)	3.1% (3/95)	9.4% (9/95)	3.1% (3/95)
	thread^{4}	5.3% (1/19)	5.3% (1/19)	5.2% (1/19)	5.2% (1/19)
	thread^{5}	5.4% (3/55)	0% (0/55)	3.6% (6/55)	3.6% (2/55)
	p-value	n.s.	n.s.		
2	Wild type	65.3% (62/95)	50.5% (48/95)	77.8% (74/95)	45.2% (43/95)
	thread^{4}	26.1% (6/23)	21.7% (5/23)	8.7% (2/23)	8.7% (2/23)
	thread^{5}	33.8% (25/74)	5.3% (4/74)	8.1% (6/74)	6.7% (5/74)
	p-value	p<0.0001	p<0.0001		
4	Wild type	59.3% (32/53)	35.2% (19/53)	64.1% (34/53)	39.6% (21/53)
	thread^{4}	12.5% (2/16)	0% (0/16)	18.7% (3/16)	6.2% (1/16)
	thread^{5}	15.4% (4/26)	0% (0/26)	3.8% (1/26)	7.7% (2/26)
	p-value	p=0.001	p<0.0001		
6	Wild type	52.0% (26/51)	34.0% (17/51)	55% (28/51)	53% (27/51)
	thread^{5}	13.5% (5/37)	2.6% (1/37)	5.4% (2/37)	8% (3/37)
	p-value	p=0.0002	p=0.0003		
8	Wild type	52.1% (25/47)	22.9% (11/47)	55% (26/47)	34% (16/47)
	thread^{5}	8.9% (5/55)	1.8% (1/55)	3.6% (2/55)	1.8% (1/55)
	p-value	p<0.0001	p=0.0011		
10	Wild type	47.4% (9/19)	15.8% (3/19)	52.6% (10/19)	10.5% (2/19)
	thread^{4}	4% (1/23)	0% (0/23)	4% (1/23)	0% (0/23)
	p-value	p=0.0023	n.s.		

Figures

Figure 2.1

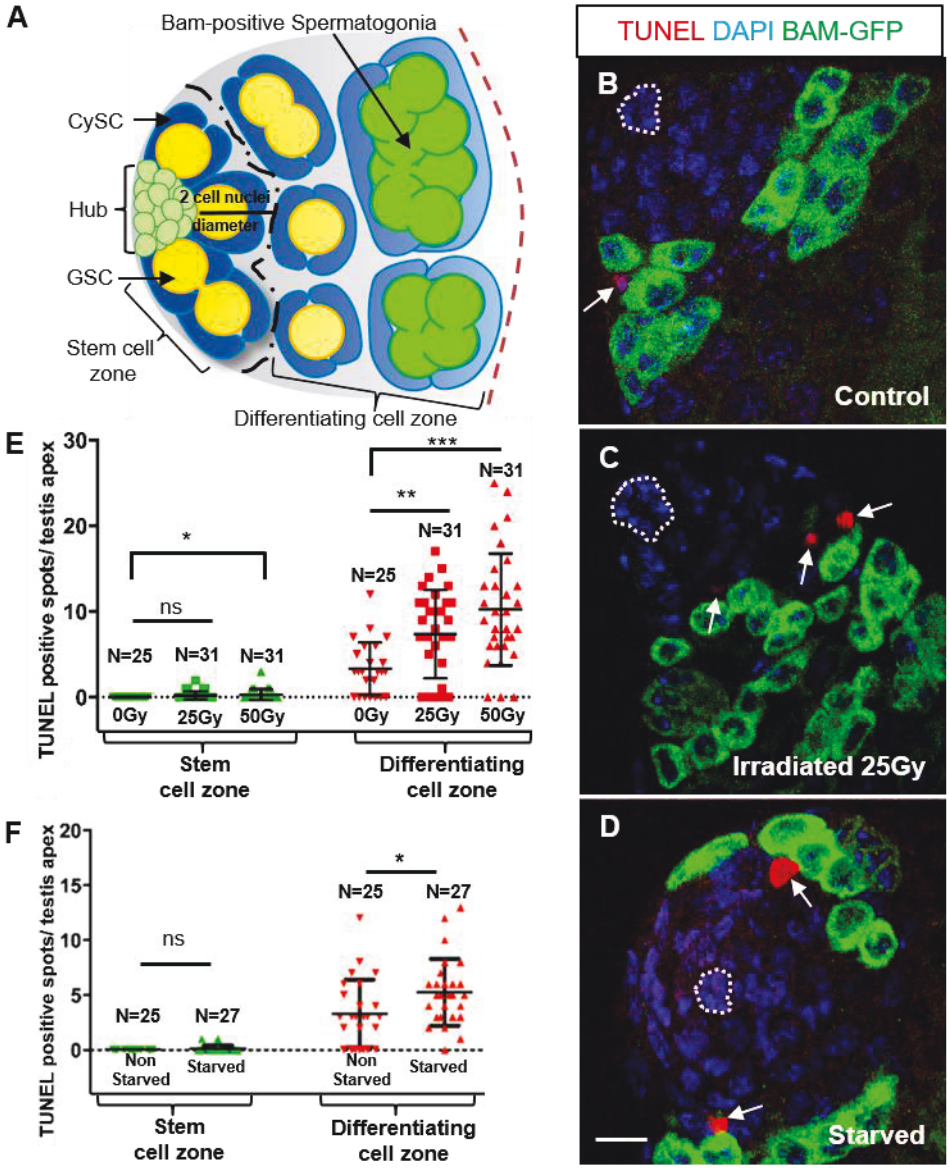


Figure 2.2

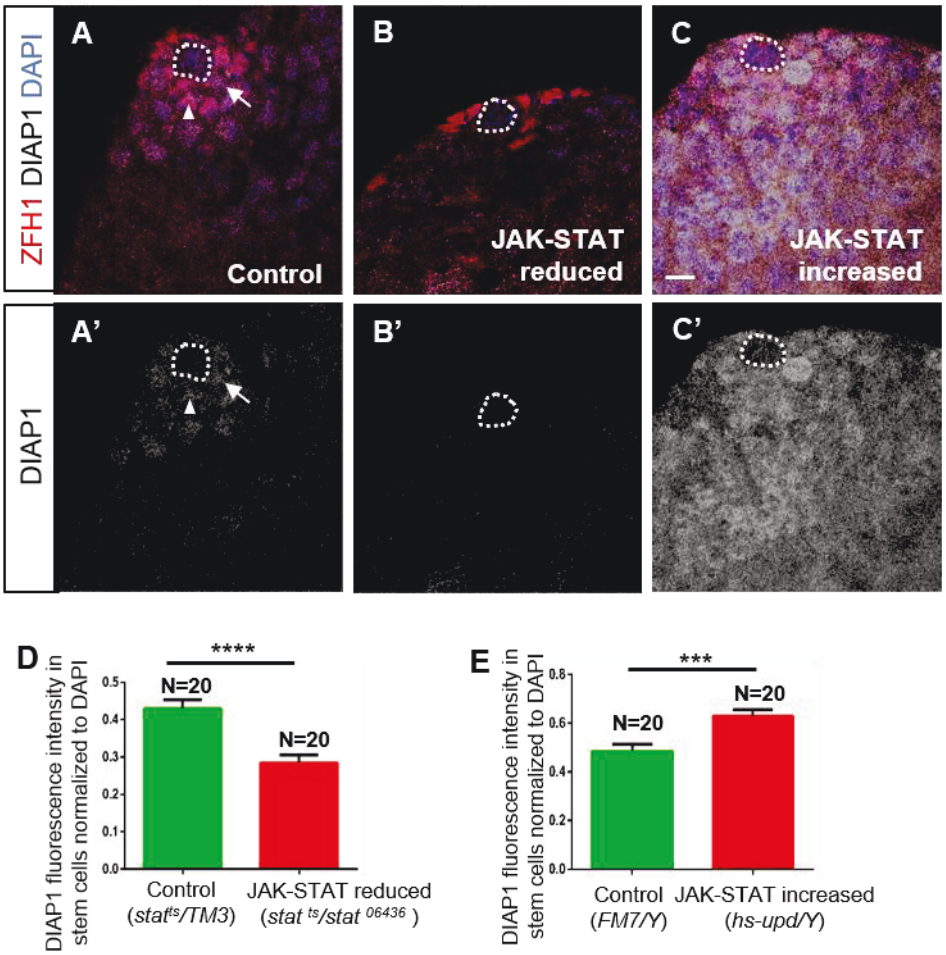

ZFH1 DIAP1 DAPI
A
Control
B
JAK-STAT reduced
C
JAK-STAT increased
DIAP1
A'
B'
C'
D
DIAP1 fluorescence intensity in stem cells normalized to DAPI
****
N=20
N=20
Control (stat^ts/TM3)
JAK-STAT reduced (stat ^ts/stat 06436)
E
DIAP1 fluorescence intensity in stem cells normalized to DAPI
***
N=20
N=20
Control (FM7/Y)
JAK-STAT increased (hs-upd/Y)

Figure 2.3

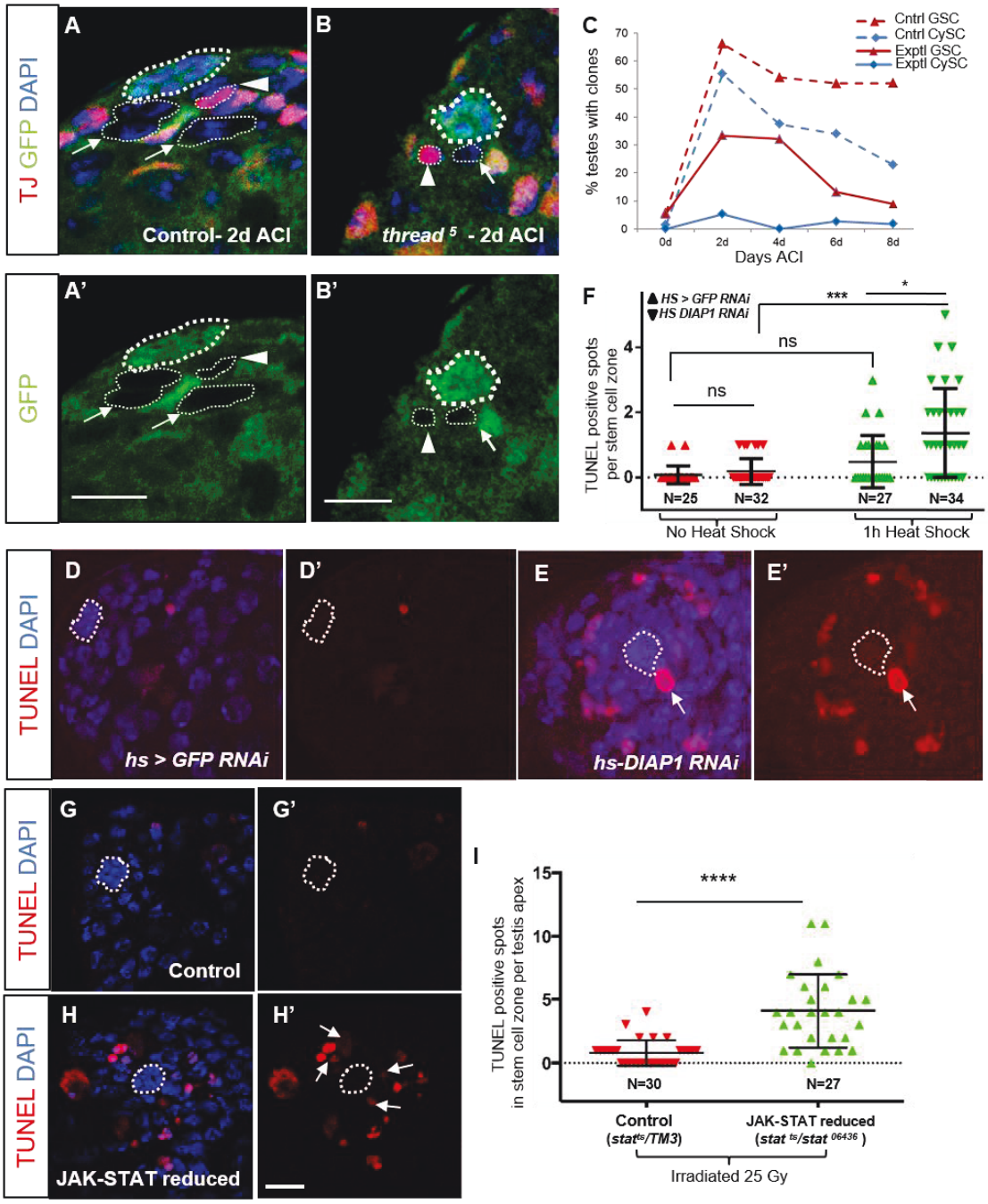

TJ GFP DAPI
A
B
Control- 2d ACI
thread 5 - 2d ACI
C
Cntrl GSC
Cntrl CySC
Exptl GSC
Exptl CySC
% testes with clones
Days ACI
GFP
A'
B'
F
HS > GFP RNAi
HS DIAP1 RNAi
TUNEL positive spots per stem cell zone
ns
ns
***
*
N=25
N=32
N=27
N=34
No Heat Shock
1h Heat Shock
TUNEL DAPI
D
D'
E
E'
hs > GFP RNAi
hs-DIAP1 RNAi
G
G'
Control
H
H'
JAK-STAT reduced
I
****
TUNEL positive spots in stem cell zone per testis apex
N=30
N=27
Control (stat ts/TM3)
JAK-STAT reduced (stat ts/stat 06436)
Irradiated 25 Gy

Figure 2.4

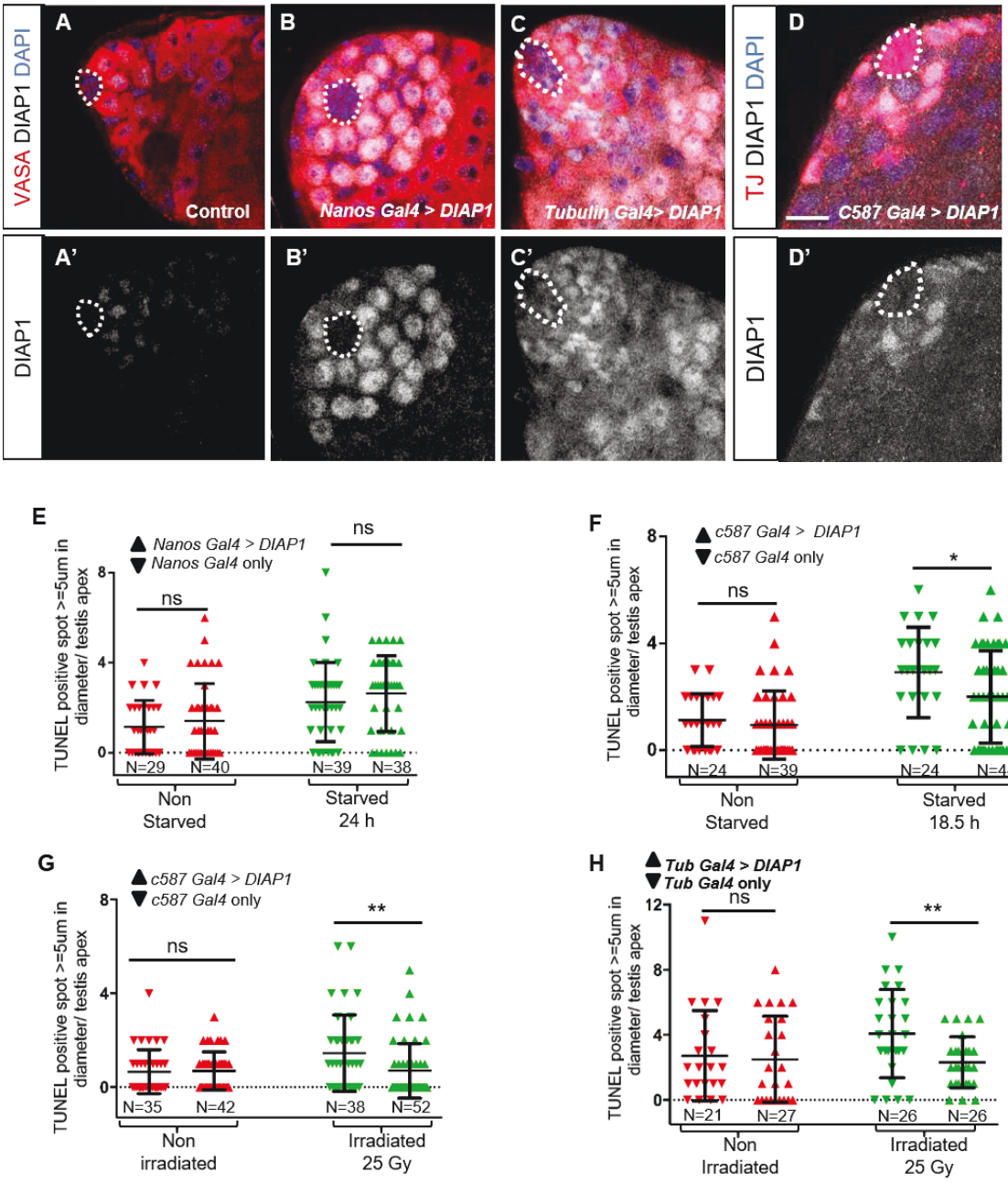
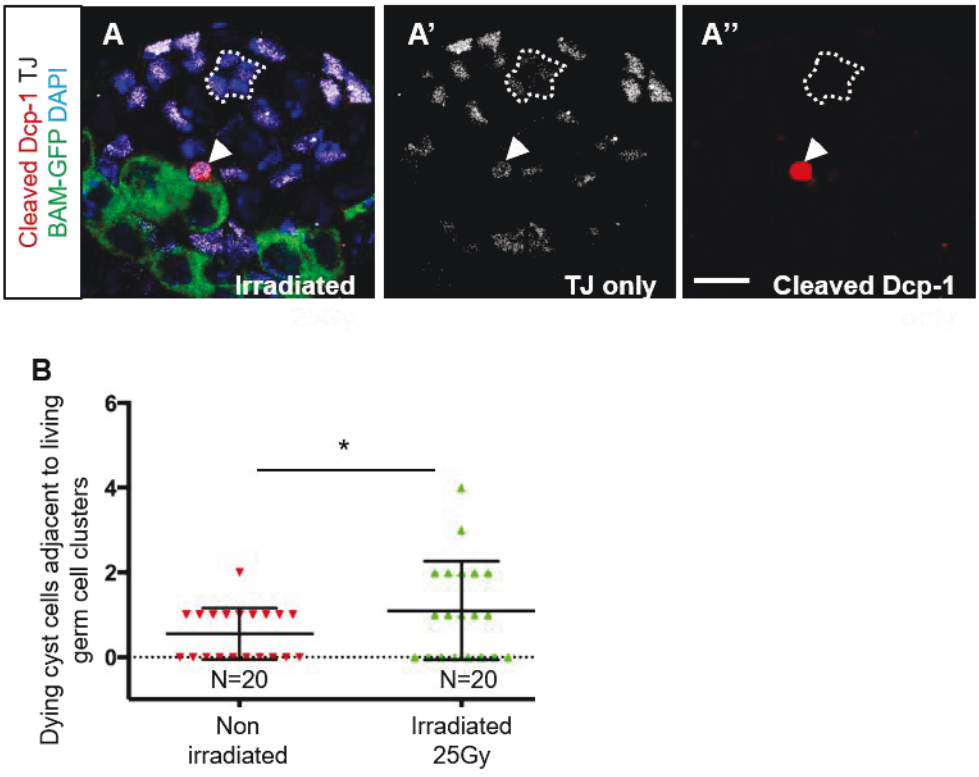

VASA DIAP1 DAPI
A
Control
B
Nanos Gal4 > DIAP1
C
Tubulin Gal4> DIAP1
TJ DIAP1 DAPI
D
C587 Gal4 > DIAP1
DIAP1
A'
B'
C'
DIAP1
D'
E
Nanos Gal4 > DIAP1
Nanos Gal4 only
TUNEL positive spot >=5um in diameter/ testis apex
ns
ns
N=29
N=40
N=39
N=38
Non Starved
Starved 24 h
F
c587 Gal4 > DIAP1
c587 Gal4 only
*
ns
N=24
N=39
N=24
N=44
Non Starved
Starved 18.5 h
G
c587 Gal4 > DIAP1
c587 Gal4 only
**
ns
N=35
N=42
N=38
N=52
Non irradiated
Irradiated 25 Gy
H
Tub Gal4 > DIAP1
Tub Gal4 only
ns
**
N=21
N=27
N=26
N=26
Non Irradiated
Irradiated 25 Gy

Figure 2.5


Cleaved Dcp-1 TJ
BAM-GFP DAPI
A
A'
A''
Irradiated
TJ only
Cleaved Dcp-1
B
Dying cyst cells adjacent to living germ cell clusters
6
4
2
0
*
N=20
N=20
Non irradiated
Irradiated 25Gy

Supplementary Figures

Figure S2.1

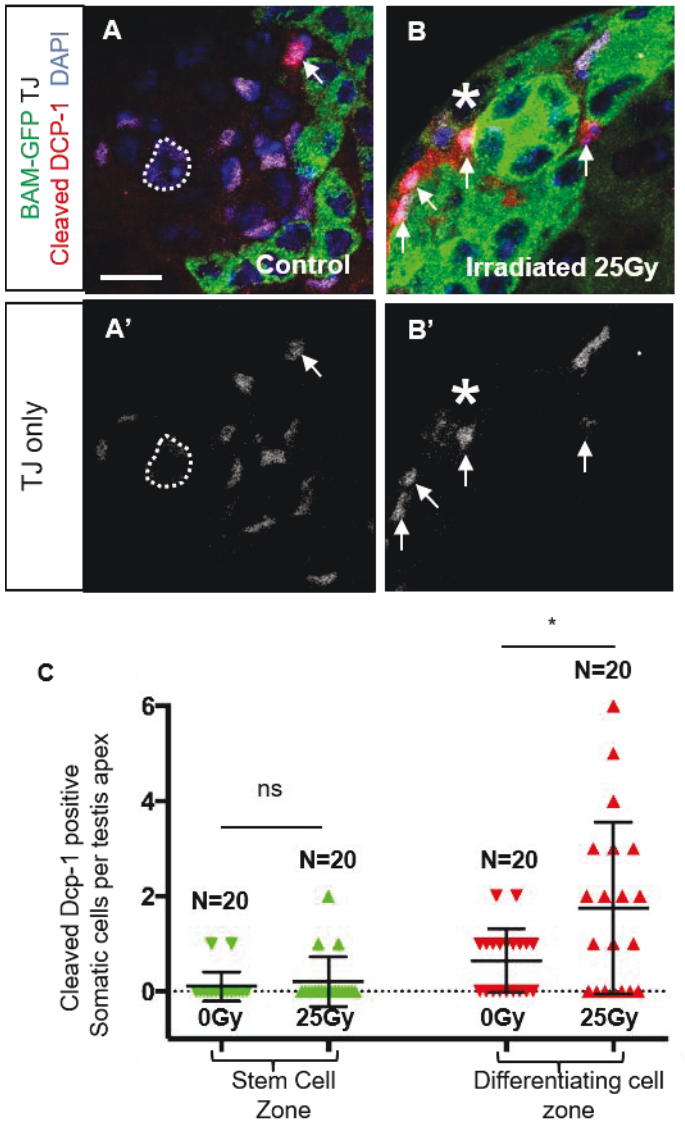


Figure S2.2

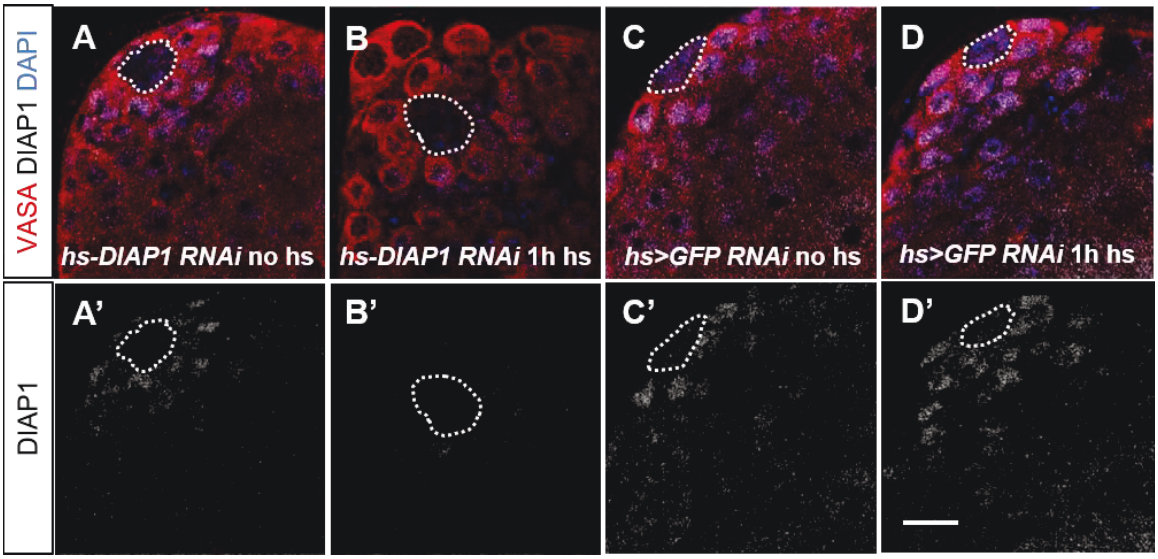

VASA DIAP1 DAPI
A
B
C
D
hs-DIAP1 RNAi no hs
hs-DIAP1 RNAi 1h hs
hs>GFP RNAi no hs
hs>GFP RNAi 1h hs
DIAP1
A'
B'
C'
D'

Figure S2.3

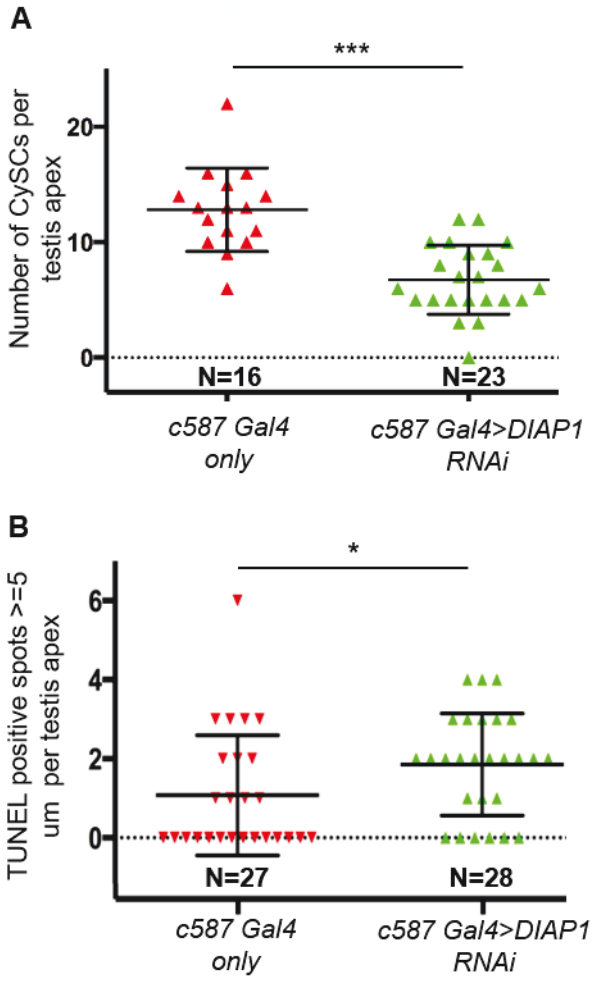
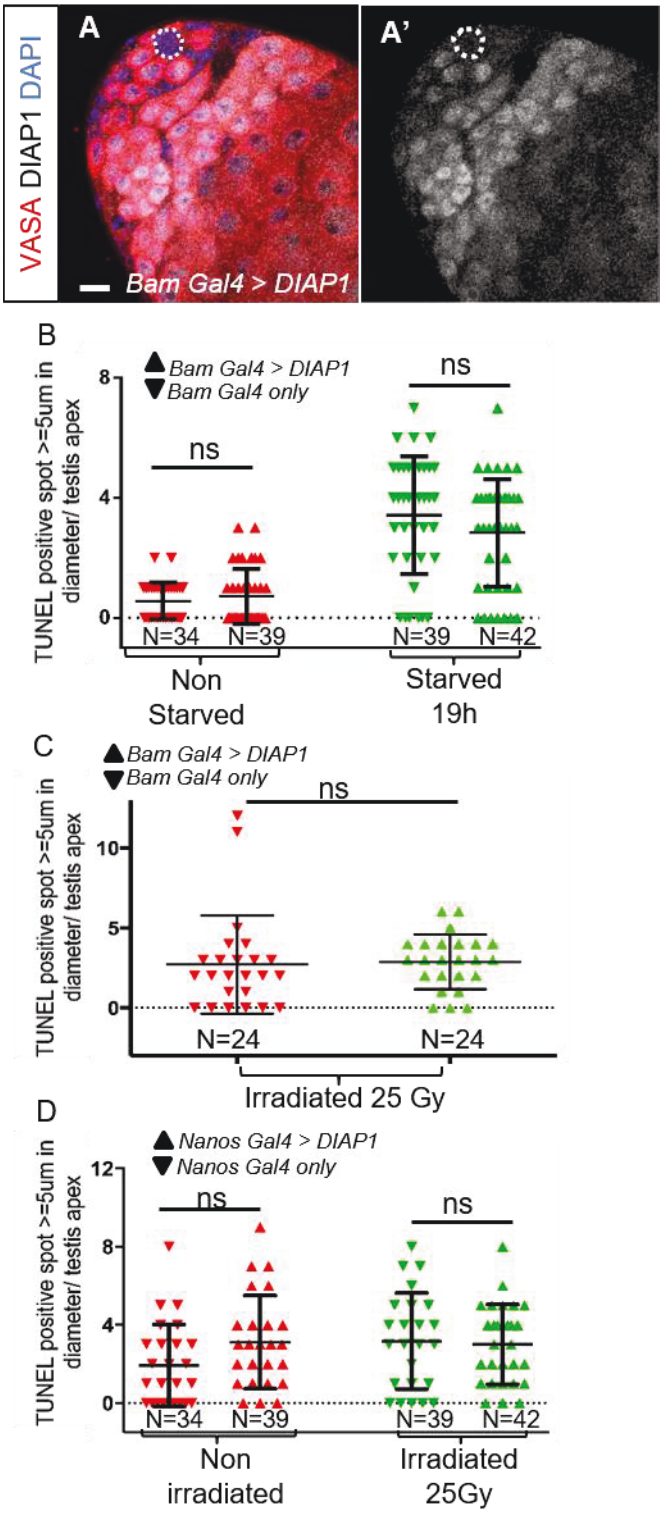

A
***
Number of CySCs per testis apex
20
10
0
N=16
N=23
c587 Gal4 only
c587 Gal4>DIAP1 RNAi
B
*
TUNEL positive spots >=5 um per testis apex
6
4
2
0
N=27
N=28
c587 Gal4 only
c587 Gal4>DIAP1 RNAi

Figure S2.4


A
A'
VASA DIAP1 DAPI
Bam Gal4 > DIAP1
B
Bam Gal4 > DIAP1
Bam Gal4 only
TUNEL positive spot >=5um in diameter/ testis apex
ns
ns
N=34
N=39
N=39
N=42
Non Starved
Starved 19h
C
Bam Gal4 > DIAP1
Bam Gal4 only
TUNEL positive spot >=5um in diameter/ testis apex
ns
N=24
N=24
Irradiated 25 Gy
D
Nanos Gal4 > DIAP1
Nanos Gal4 only
TUNEL positive spot >=5um in diameter/ testis apex
ns
ns
N=34
N=39
N=39
N=42
Non irradiated
Irradiated 25Gy

Chapter 3

Homologous recombination is essential for DNA damage-induced regeneration of germline stem cells in the Drosophila testis niche

This chapter is a modified version of the manuscript - Hasan, S., Matunis, E.L., 2017. Homologous recombination is essential for DNA damage-induced regeneration of germline stem cells in the Drosophila testis niche (In preparation).

Summary

Stem cells have been shown to survive radiation-induced DNA damage in many adult tissues. However, the exact pathways which are used by these cells to repair DNA damage in an intact tissue are not very well-understood. Here we use the Drosophila testis niche to understand the processes of DNA damage response and repair in an intact niche. Historical studies of DNA damage in Germline stem cells (GSCs) in the Drosophila testis niche have shown that GSCs preferentially survive IR, while their differentiating progeny do not. The radio-resistance of stem cells suggested they efficiently use DNA double-strand break (DSB) repair pathways, but this had not been examined directly. To study the effects of DNA DSB repair in GSCs in an intact stem cell niche, we establish conditions to induce a level of DNA DSBs that eliminates half of the GSCs in the adult Drosophila testis. Clonal analysis suggests that GSCs are eliminated by detachment from the niche. Surviving GSCs repopulate the niche, indicating they can repair DNA damage and resume function. In contrast, GSCs lacking the highly conserved kinase and DNA repair pathway component Checkpoint kinase 2 (Chk2) and its target p53 do not demonstrate any GSC loss upon IR, but eventually all die, indicating that as is the case in many other cells, these canonical DNA repair pathway genes are necessary for GSCs to sense and repair DNA DSBs. p53 activates two major DNA DSB repair pathways - Non homologous end joining (NHEJ) and homologous recombination (HR), which are typically functionally redundant, and not required for organismal or cell viability under physiological conditions. Therefore, we asked how GSCs lacking members of each pathway respond to DNA DSBs. We found that the response of GSCs to IR in flies

null for Lig4, which are unable to repair DNA via NHEJ, is phenotypically indistinguishable from wild type (WT), indicating that NHEJ is not required for damage-induced regeneration. In contrast, GSCs in flies lacking Blm or Rad51, which are unable to execute HR, are more sensitive to IR, and fail to regenerate the GSC pool over time. This indicates that HR, but not NHEJ, is required for GSCs to regenerate the GSC pool post IR. This contrasts with many other cells, which can use either of these pathways to repair DNA DSBs. Instead, in GSCs an interesting mechanism exists to ensure that the more accurate form of DNA repair (HR) is promoted over the error prone NHEJ.

Introduction

Tissue specific adult stem cells are found in many different organisms and are responsible for long term maintenance of tissues (reviewed in Rossi et al., 2008). Stem cells are typically found in a specific microenvironment called the stem cell "niche". The niche is regulated by specific niche cells which secrete factors and signals that help in maintenance and survival of the stem cells (Jones and Wagers, 2008). Stem cells, like other cells in an organism, are exposed to multiple stresses such as nutrient deprivation and genotoxic damage (reviewed in Mandal et al., 2011; McLeod et al., 2010). Nutrient deprivation (lack of protein) can lead to reversible loss of GSCs in adult Drosophila testes and ovary (de Cuevas and Matunis, 2011a). Genotoxic stress can include any stress that damages DNA. Genotoxic stress can arise from both endogenous and exogenous insults (reviewed in Helleday et al., 2014). While endogenous genotoxic stresses can include reactive oxygen species and replication fork collapse, exogenous sources of genotoxic stress include radiation – ionizing or ultraviolet and chemical agents like chemotherapeutic drugs (reviewed in Helleday et al., 2014). As an exogenous source of genotoxic stress, ionizing radiation (IR) can be very damaging to a cell because it induces of DNA double-strand breaks (DSBs) (reviewed in Schwertman et al., 2016). DNA DSBs can be harmful to cells for two main reasons – first, cells cannot replicate DNA or divide without repairing DSBs in their DNA. Second, improper repair of DNA DSBs can lead to potentially harmful mutations (reviewed in Schwertman et al., 2016).

The molecular mechanisms for sensing and repairing IR-induced DNA DSBs are highly conserved. Phosphorylation of Histone 2A variant (H2AX in mammals, or

H2AvD in Drosophila) results in activation of the Checkpoint kinase (Chk2), which phosphorylates the tumor suppressor p53 (reviewed in Goodarzi and Jeggo, 2013). Both activated Chk2 and p53 promote cell cycle arrest, providing the cell time to repair DNA DSBs (reviewed in Goodarzi and Jeggo, 2013) . Cells typically remain arrested until DSBs are repaired, after which the cells can resume proliferation. If DNA damage is too severe the cell initiates programmed cell death (reviewed in Goodarzi and Jeggo, 2013).

DNA DSBs are repaired mainly through two pathways – Non-homologous end joining (NHEJ) or Homologous recombination (HR) (reviewed in Goodarzi and Jeggo, 2013). These pathways are not essential for viability, since they are often functionally redundant (Johnson-Schlitz et al., 2007). However, each pathway relies on the activity of a distinct set of genes, and the outcomes following repair also differ at the molecular level (Johnson-Schlitz et al., 2007). HR is typically non-mutagenic as it requires a template DNA strand. However, this means it can only occur during the S or G2 phase of the cell cycle (reviewed in Goodarzi and Jeggo, 2013). In contrast, NHEJ often introduces changes to the DNA sequence (such as deletions). NHEJ, unlike HR, can occur at any phase of the cell cycle but predominates during G1 (reviewed in Lieber, 2010). NHEJ is known to occur more quickly than HR (Mao et al., 2008), and NHEJ is thought to be the primary mechanism by which quiescent cells repair DNA damage (reviewed in Iyama and Wilson, 2013).

Different proteins are required for NHEJ and HR pathways to successfully occur in cells. The HR pathway involves several conserved proteins including Rad54, Rad51and Bloom syndrome helicase protein (Blm). Rad54 and Rad51 are necessary

for pairing the DNA strand containing the DSB to its homologous DNA strand in order to synthesize the broken segment in the DNA (reviewed in Chapman et al., 2012). The Blm protein was initially thought to support the NHEJ pathway in studies done in Drosophila L2 culture cells (Min et al., 2004). However, detailed work by Johnson-Schlitz et al., showed that Blm is involved in dissolution of double-Holliday junctions during the HR repair in germ cells of the Drosophila adult testis (Johnson-Schlitz and Engels, 2006). Similarly, Blm has been shown to mediate HR repair in cultured human fibroblasts (Bischof et al., 2001). The NHEJ pathway is known to employ Ku70/80 and DNA ligase IV (Lig4) proteins that bind to the broken ends of DNA during DSB repair. Lig4 ligates the broken end of DNA during NHEJ repair (reviewed in Lieber, 2010).

Most of what is known about DNA damage repair in stem cells comes mainly from studies of cultured Hematopoietic stem cells (HSCs) (reviewed in Li et al., 2016). Upon irradiation, quiescent HSCs use NHEJ to repair their DNA (Mohrin et al., 2010b). However, when HSCs are forced into proliferation and then irradiated, they use HR. In contrast, much less is known about how other types of adult stem cells, particularly those within intact tissues, respond to DNA damage, but it is clear that the responses are cell-type dependent. For instance, in mice, a dose of IR sufficient to drive intestinal stem cells (ISC) into apoptosis does not ablate HSCs (reviewed in Blanpain et al., 2011)(Blanpain et al., 2011b). Interestingly, this differential response is not because HSCs are quiescent while ISCs are actively proliferating but due to the reduced expression of anti-apoptotic and DNA repair proteins in ISCs compared to HSCs (reviewed in Blanpain et al., 2011). Less is

known about the DNA damage response in the germline, where one would predict that a non-mutagenic mechanism such as HR would be preferred. However, it is thought that the long G1 phase in mouse GSCs (measured using fluorescent ubiquitin cell cycle indicator (FUCCI)) could promote NHEJ use (Ishii et al., 2014b). Indeed, one study using adult mice mutant for canonical NHEJ repair suggested that GSCs in the adult mouse testis use an alternative, more error-prone form of NHEJ to repair IR-induced DSBs (Rübe et al., 2011).

Due to its simple cellular arrangement and multitude of genetic tools, the well-understood model system of the Drosophila testis can be used to understand DSB repair in GSCs. The adult Drosophila testis apex houses the GSCs which are arranged around a group of non-mitotic cells called "the hub" (Fig. 3.1A) (reviewed in Greenspan et al., 2015). Each GSC is flanked by two Cyst stem cells (CySCs) (Fig. 3.1A). The GSCs undergo mitosis to produce a single differentiating daughter called "the gonialblast". The gonialblast undergoes multiple rounds of incomplete, transit-amplifying divisions to produce interconnected germ cell clusters called "spermatogonia" (Fig. 3.1A) (reviewed in Greenspan et al., 2015). The spermatogonia gives rise to spermatocytes which undergo meiosis to produce sperm. The GSCs also have a special organelle called the fusome. The fusome has a spherical appearance in the GSCs and the immediate daughters but takes on a more "branched" morphology in the spermatogonia (Fig. 3.1A) (reviewed in Greenspan et al., 2015).

The hub is known to produce signals that help in maintenance of the GSCs. One such signal is Unpaired which activates the Janus kinase-Signal transducer and

activator of transcription (JAK-STAT) pathway in the GSCs and CySCs (Kiger et al., 2001; reviewed in Stine and Matunis, 2013; Tulina and Matunis, 2001). In our previous work, we found that niche signals promote the expression of anti-apoptotic protein Drosophila inhibitor of apoptosis 1 (DIAP1) in GSCs and CySCs through the JAK-STAT pathway (Hasan et al., 2015). Expression of DIAP1 in GSCs and CySCs protects them from IR-induced cell death. The spermatogonia which do not receive signals from the niche, express comparatively reduced levels of DIAP1 and undergo cell death in response to IR (Hasan et al., 2015). Since IR is known to cause DNA DSBs (reviewed in Mladenov and Iliakis, 2011), the radio resistance of stem cells suggests they efficiently use DNA DSB repair pathways.

The roles of DNA damage response pathway members have been studied in the Drosophila ovary, showing that members of the HR pathway are essential for meiotic recombination (reviewed in Ables, 2015; McKim et al., 2002). In contrast, little is known about use of DNA damage repair pathway members in the ovarian stem cells. For example, female GSCs activate the canonical DNA damage response pathway member Chk2 in response to IR (Ma et al., 2016), but how this DNA damage is repaired is not known. Similarly, the DNA damage repair pathways used by male GSCs in Drosophila testis have not been studied. However, Preston et al., 2006 surveyed pathway selection in spermatocytes, finding that these cells initially preferred NHEJ over HR in young males, but that this preference shifted to HR with age (Preston et al., 2006). At any given point in time, fly testis GSCs are either in G2 (75% of GSCs) or S phase of cell cycle (21% of GSCs) with only 3.8% of GSCs in G1 and 0.2% in M (Sheng and Matunis, 2011). Thus, both NHEJ and HR should be

available to these cells most of the time. The availability of both HR and NHEJ to repair DNA in majority of fly GSCs, presents an excellent system to study the impact of DNA damage repair pathway choice in response to IR and the factors that might regulate it. Here we investigate the pathways that are involved in DNA DSB repair in GSCs by analyzing GSC regeneration after a high dose of IR eliminates half of them.

Results

Establishing conditions to study DNA damage-induced Germline stem cell (GSC) regeneration in vivo

In order to establish conditions to study the response of Drosophila male GSCs to DNA damage, we first sought to characterize the dose-response of these cells to IR. We exposed adult males to increasing doses of IR, then dissected, fixed and immuno-stained their testes to determine the effects on GSCs. We previously found that GSCs in the adult Drosophila testis are resistant to IR at low doses of 25 Gy (Hasan et al., 2015) and 50 Gy (Xing et al., 2015). Therefore, we chose to use radiation doses of 50, 75 and 100 Gy. To confirm that these doses of IR induce DNA damage in all cells in the testis, as we have seen occurs following lower doses of IR (Hasan et al., 2015), we immuno-stained testes from irradiated males with anti-sera raised against γH2AvD, which labels DNA repair foci in multiple Drosophila tissues including the adult testis (Delabaere et al., 2016). Robust γH2AvD signals are visible in the nuclei of all cells of within the testis apex within 1-2 hours post-IR (Supp. Fig. S3.1). Expectedly, the intensity of yH2AvD immuno-stain increases with increasing radiation dose demonstrating an increase in DNA damage (Supp. Fig. S3.1). To quantify IR-induced loss of GSCs, testes were immuno-stained with anti-sera raised against the germ cell protein Vasa and specific germ cell structure fusome protein Hu-li tai shao (Hts) (Matunis et al., 2012). GSCs were counted in each testes as $Vasa^{+}$ cells with spherical fusome next to the hub in each testis. Since cell death can be detected within 3 hours of exposure to IR (Hasan et al., 2015), we hypothesized that assaying testes one day post-IR could be sufficient time to allow IR-induced loss of

GSCs to occur. One day post 50 Gy IR, no loss of GSCs was apparent compared to un-irradiated controls (7.6 versus 7.8 GSCs/testis, N=26-28 testes) (Fig 3.1B, C, H), as expected (Xing et al., 2015). Higher doses of IR caused a decrease in GSC numbers. By 24 hours after exposure to 75 Gy IR, the GSC number dropped significantly to an average of 3.5 GSCs/testis (N=22, $p<0.001$) (Fig 3.1D, H). A similar reduction in GSC number was observed one day after exposure to 100 Gy IR (3.25 GSCs/testis, N=16, $p<0.001$) (Fig 3.1F, H). In addition to effects on GSCs, we also observed an almost complete loss of differentiating germ cells at 24 hours post-IR at all three radiation doses (50, 75 and 100 Gy) (Fig 3.1C, D, F). This was expected since we and others have previously shown that differentiating germ cells do not survive (25 or 50 Gy) of IR (Hasan et al., 2015; Xing et al., 2015). We conclude that irradiating flies with 75 and 100 Gy, but not 50 Gy, is sufficient to remove part of the GSCs from the niche within 24 hours post irradiation.

To ask whether GSCs surviving 75 or 100 Gy of IR will eventually die, due to irreparable DNA damage, or instead repair this damage, resume divisions, and repopulate the niche, we counted GSCs one week post-IR. We found an average of 7.71 GSCs/testis (N=27 testes) after one week in males exposed to 75 Gy IR which was similar to GSC number in un-irradiated controls (average of 7.76 GSCs/testis, N=26 testes) (Fig 3.1E, H). In contrast, the number of GSCs was reduced significantly to an average of 2.1 GSCs/testis (N=28) one week after exposure to 100 Gy compared to un-irradiated controls ($p<0.001$), with most (71%) of the testes from irradiated flies lacking all GSCs (Fig 3.1F, G, H). Our results at 100 Gy matched the

qualitative results regarding the fertility of adult Drosophila males irradiated with 100 Gy IR published historically (Welshons and Russel, 1957).

It is interesting to note that while testes look similar one day after exposure to 75 or 100 Gy in terms of GSC number (Fig. 3.1D, E, H), our results above indicate that 75 Gy, but not 100 Gy, is tolerated by GSCs (Fig 3.1F-H). Our results also suggest that in GSCs, the cellular decision to die or to complete DNA repair and divide occurs at about one day post-IR exposure at high doses of 75 or 100 Gy, and indicate that we have established an appropriate paradigm to study IR-induced DNA damage, stem cell loss, and regeneration in a well-defined population of GSCs in vivo.

GSCs leave the niche instead of dying there post 75 Gy IR

Loss of GSCs upon IR could represent cell death or differentiation. To distinguish between these possibilities, we immuno-stained testes with TUNEL (Terminal dUTP nick end labeling), to identify apoptotic cells. TUNEL staining has been previously been used to identify dying germ cells death in both un-irradiated and irradiated testes (Hasan et al., 2015; Yacobi-Sharon et al., 2013). Under physiological conditions, TUNEL-positive cells are not observed adjacent to the hub, suggesting GSCs are protected from cell death in the niche (Hasan et al., 2015) (Fig. 3.2A, C) (0 dying GSCs in N=20 testes). Interestingly, TUNEL-positive cells were also not evident next to the hub at 3 hours post-IR (Fig. 3.2B, C) (0.06 dying GSCs in N=16 testes), suggesting that GSC do not die in this location at this time-point. TUNEL staining was working as expected, however, since an elevated number of TUNEL-positive

cells were observed away from hub, representing dying spermatogonia (Fig. 3.2B, C arrows).

The absence of TUNEL-positive GSCs does not indicate that these cells never die. For instance, GSCs could die more slowly than differentiating germ cells, (e.g. if 3 hours is not the ideal time to look for GSC apoptosis) we may have missed it. Therefore, we genetically suppressed the apoptotic pathway by over-expressing the caspase inhibitor p35 in GSCs(Ma et al., 2016). We reasoned that if GSC loss is due to apoptosis then p35 over expression should lead to rescue of GSC loss. Since there is no available anti-sera for p35, we used a modified version of p35 which has been tagged with the octa-peptide FLAG (p35-FLAG) to confirm that over-expression of p35 occurred as expected (Ma et al., 2016). p35-FLAG has been previously shown to have similar functionality as untagged p35 in GSCs in adult Drosophila ovary (Ma et al., 2016). Using a Nanos-Gal4 driver, we were able to over-express p35-FLAG in the GSCs and their immediate daughters in adult testes (Fig. 3.2I, J) (reviewed in White-Cooper, 2012). However, over-expressing p35 in GSCs did not prevent their loss one day post-IR exposure (Fig 3.2D-G, H), consistent with the hypothesis that apoptosis is not the primary cause of GSC loss upon IR.

Formally, it is possible that a non-apoptotic cell death may play some role in IR-induced GSC loss. However, since GSC cell death in adult Drosophila testis has never been observed under physiological conditions (Hasan et al., 2015), it is not clear which non-apoptotic pathway GSCs would use and therefore should be tested. Overall, our results suggest that GSCs do not enter apoptosis within the niche after 75 Gy IR.

Based on our results, it is also possible that instead of cell death, IR-induced GSC loss is due to differentiation. If GSCs were differentiating post 75 Gy IR, then testes containing GSC clones marked with green fluorescent protein (GFP) should show a decrease in marked GSCs and an increase in marked spermatogonia post-IR. Therefore, we induced mitotic clones in GSCs using the heat-shock induced Flp-FRT (Flippase- Flippase recognition target) system (Jiang and Edgar, 2009), allowed clones to mature for 2 days induction to enable the marking to extend to older germ cells, including spermatogonia, then irradiated adult males. One day after irradiation, testes were dissected, fixed and immuno-stained to determine the number of testes containing clones, and the behavior of the clones over time. Testes containing GSC clones did not differ significantly from 36.1% testes containing GSC clones (N=36 testes) in un-irradiated controls to 26% testes containing GSC clones (N=46 testes). However, in the same time period, the number of testes containing spermatogonial clones fell significantly from 66.6% testes (N=36 testes) to 32.6% testes (N=46 testes) ($p=0.0036$, 2-tailed Fisher's exact test) after irradiation exposure, as expected based on the extensive cell death seen in this cell type. We also saw an unexpected, novel phenotype. Instead of an increase in differentiating germ cell clones, we saw multiple single germ cell clones at a distance of more than two cell nuclei diameter away from the hub. Typically, single germ cells in the testis apex are GSCs or gonialblasts and are only seen within close proximity (2 cell nuclei diameters) of the hub (reviewed in Greenspan et al., 2015). We searched for single germ cell clones in a defined "differentiating cell zone" - part of testis apex excluding first two tiers of cells adjoining the hub. As expected, single germ cell clones were not seen in

differentiating cell zone in un-irradiated testes containing spermatogonial clones (Fig. 3.3A, A'). However, in testes dissected one day after exposure to 75 Gy IR, a significantly higher, 22% of testis (10 out of 46 testes containing germ cell clones, Table 3.1) had single germ cell clones in the differentiating cell zone (Fig. 3.3A-B, C, Table 3.1). Un-irradiated testes at this time-point did not have any single germ cell clones (Fig. 3C, 0 out of 21 testes, Table 3.1). We wondered what is the fate of the single germ cell clones and if they are lost with time. Indeed, the single germ cell clones were lost 3 days after exposure to IR (Fig. 3C). The loss of single germ cell clones could be due to death or it is possible that the single germ cell clones divided. The presence of single germ cell clone can be due to GSCs that detach the hub and move away post-IR. Alternatively, they could be the remnants of an interconnected cluster of spermatogonia that fragmented (Sheng et al., 2009), perhaps due to radiation exposure. If the single germ cell clones arose from fragmentation of a marked spermatogonial cluster then we should always see even numbers of spermatogonia. For example, a 4-cell spermatogonia would break into 4 single germ cell clones. In our data, one day after radiation exposure, 70% of testes contained an odd number of single germ cell clones suggesting spermatogonia are not the source of these single marked germ cells. It is also possible that one of the cells from a spermaotgonial cluster survived while the remaining died. However, this seems unlikely since all the interconnected germ cells in a spermaotgonia are known to undergo cell death together (Hasan et al., 2015; Yacobi-Sharon et al., 2013; Yang and Yamashita, 2015).

Under physiological conditions, detachment of GSCs from the hub is rarely seen in live in ex-vivo culture of testes (Sheng and Matunis, 2011). Since our data suggested detachment of GSCs from the hub in response to IR exposure, we wondered if live imaging of irradiated testes would reveal GSCs detaching from the hub. We irradiated flies and immediately removed their testes and placed in an ex-vivo culture (see methods) (Greenspan and Matunis, 2017). The testes were then imaged live overnight for 9.5 hours. As expected, among irradiated testes we saw a significant 12% of all GSCs (8 out of 66 total GSCs across 7 testes) detaching from the niche within the imaging time frame (Fig. 3E-G). In contrast no detachment of GSCs was visible in un-irradiated controls (0 out of 59 total GSCs across 6 testes, p=0.0088, 2-tailed, Fishers exact test). The detached GSCs moved away from the niche and did not divide or return to the niche, during the entire length of the live-imaged movie. This data clearly demonstrated that GSCs detach in response to radiation exposure and are thus lost from the niche. Altogether, our data suggest that GSCs are not likely to be lost from the testis through apoptosis post 75 Gy IR. Instead, irradiation causes GSCs to detach from the hub. The detached GSCs move away from the hub and survive as single germ cells until they are lost few days later, likely through cell death.

DNA damage-induced GSC loss requires Chk2-mediated activation of p53

Next we wondered if the responses of GSCs to irradiation are mediated by the canonical DNA damage response machinery. There is precedent for this in Drosophila. Ma et al., 2016 showed that GSCs are not lost upon exposure to IR (200

Gy) in the fly ovary lacking Chk2 (Ma et al., 2016), which is a key component of the DNA damage response (reviewed in Zannini et al., 2014), but the ability of these cells to sustain regeneration after this very high dose of IR was not tested. Furthermore, the role of Chk2 in the testis was not known. We hypothesized that IR-induced GSC loss requires Chk2. Consistent with this hypothesis, testes from flies completely lacking Chk2 were phenotypically indistinguishable from wild-type testes before irradiation, as expected, and initially showed no phenotypic changes, maintaining a normal number of GSCs, upon 75 Gy of IR (average of 8.3 GSCs/testis, N=28 testes), compared to irradiated heterozygous controls (average of 5.4 GSCs/testis, N=20), which lose 50% of GSCs under these conditions (Fig. 3.4 A-D, G). We conclude that Chk2 mediates the sensing of DNA damage in GSCs. Since p53 is a direct target of Chk2-dependent activation (Brodsky et al., 2004), we hypothesized that GSCs lacking Chk2 will also not lose GSCs under these conditions. Consistent with this hypothesis, testes from $p53^{-/-}$ flies subjected to 75 Gy IR were indistinguishable from those lacking Chk2 (average of 10.68 GSCs/testes, N=32 testes, Fig. 3.4 H-K, N). To determine if Chk2 and p53 are required cell-autonomously in the GSCs to mediate DNA damage-induced GSC loss and subsequent regeneration, we knocked down Chk2 specifically in GSCs and their immediate daughters using Chk2 RNAi driven by Nanos Gal4. Since Chk2 null flies do not show any developmental abnormality (Fig. 3.4 A-B, G) with respect to GSCs, we did not block Gal4 activity by using $Gal80^{ts}$ (Suster et al., 2004) in knocking down Chk2. Similar to $Chk2^{-/-}$ flies, flies expressing Chk2 RNAi in GSCs showed no phenotypic change upon exposure to 75 Gy IR and the number of GSCs per testis remained similar to that seen in un-

irradiated controls (Fig. 3.5 D, E, J). We conclude that GSCs autonomously require Chk2 to sense DNA damage. The same is true for p53: GSC specific knockdown of p53 produces similar results. To reduce functional p53 in GSCs, we over-expressed a dominant-negative mutant allele of p53 ($p53^{R155H}$) using Nanos-Gal4. The dominant-negative mutant $p53^{R155H}$ is known to reduce the amount of functional p53 available in the cell by forming inactive complexes that do not bind to DNA and therefore, abolish the transcriptional activity of p53 (Ollmann et al., 2000). Expression of $p53^{R155H}$ in the adult Drosophila eye suppresses irradiation-induced apoptosis (Ollmann et al., 2000). Over-expression of this allele in GSCs throughout development does not give any phenotype in the adult males under normal conditions (Monk et al., 2012) (Fig. 3.5 D, F, J). Similar to flies completely lacking p53, GSC specific reduction of functional p53 showed no significant change in GSC number (average of 8.91 GSCs/testes, N=17 testes) compared to un-irradiated controls (average of 9.3 GSCs/testes, N= 28 testes) (Fig. 3.5 J). Since Nanos Gal4 expression is completely reduced by 8 cell spermatogonia (Yacobi-Sharon et al., 2013), loss of some spermatogonia was obvious in both Chk2 RNAi and $p53^{R155H}$ expressing testes (Fig. 3.5 D-F). Altogether, our data suggest that all GSCs autonomously activate the canonical DNA damage response pathway involving Chk2 and p53 post-IR and the decision of some stem cells to detach or remain in the niche upon IR is likely due to a processes downstream of p53 activation.

Chk2-dependent activation of p53 is required for long term maintenance of GSCs after exposure to irradiation

Since activation of Chk2 is usually associated with activation of DNA damage repair via p53 (Brodsky et al., 2004), we wondered if GSCs in flies lacking Chk2 would eventually be lost since they are burdened with unrepaired DNA damage. Alternatively, there could be Chk2-independent activation of DNA damage repair, which would subsequently enable GSCs to resume proliferation and tissue regeneration. In order to test this, we examined $Chk2^{-/-}$ testis 1 week post 75 Gy IR. Interestingly, we found significant loss of GSCs in $Chk2^{-/-}$ testes a week after IR (a mean of 5.88 GSC/testis, N= 25 compared to 10.68 GSC/testis, N=16 in irradiated heterozygous controls, $p<0.0001$) (Fig. 3.4 E-F, G). As seen in $Chk2^{-/-}$ testes, GSC-specific reduction of Chk2 by expressing Chk2 RNAi in the germline using Nanos-Gal4 significantly reduced the number of GSCs one week after radiation exposure (Fig. 3.5 G-H, K). This indicates that Chk2 is required for long-term survival of irradiated GSCs.

Since p53 is down stream of Chk2 activation (Brodsky et al., 2004), we wondered if GSCs in $p53^{-/-}$ flies would also eventually be lost as well because of unrepaired DNA damage. Alternatively, it is possible that there exists Chk2-dependent activation of DNA damage repair which does not require p53, resulting in proliferating GSCs in testes lacking p53. To distinguish between these possibilities, we examined $p53^{-/-}$ testis 1 week post 75 Gy IR. Interestingly, we found a near complete loss of GSCs in $p53^{-/-}$ testes a week after IR (a mean of 1 GSC/testis, N=18 compared to 10.61 GSC/testis, N=32 in heterozygous controls, $p<0.001$) (Fig. 3.4 L-M, N). Furthermore, GSC specific reduction of functional p53 by over-expressing dominant-negative $p53^{R155H}$ significantly reduced the number of GSCs to an average

of 4.25 GSCs/testes, (N=12 testes) compared to an average of 7.2 GSCs/testes (N=23 testes, $p<0.05$) in controls, one week after radiation exposure (Fig. 3.5 G, I, K), indicating that GSCs autonomously require p53 following a 75 Gy dose of IR.

Together, these results indicate that although GSCs in animals lacking Chk2 or p53 temporarily survive IR-induced damage, Chk2-dependent activation of p53 is required to enable the long-term maintenance of GSCs post-IR.

DNA DSB repair is p53-depenedent in the GSCs and the hub

Typically, p53 activation stalls the cell cycle to allow cells time to repair DNA damage (reviewed in Reinhardt and Schumacher, 2012). In order for successful regeneration of lost GSCs by the GSCs that remain at the hub after radiation exposure, successful DNA DSB repair is required. We wondered if cells lacking p53 can undergo successful DNA DSB repair at a rate similar to controls. While the role of p53 in cell cycle arrest post-IR exposure in mammalian cell lines as well as mouse models is well known (reviewed in Reinhardt and Schumacher, 2012), it is possible that activation of DNA DSB repair pathway does not solely depend on activation of p53. Indeed, by using γH2AvD as a marker for un-repaired DNA DSBs, Wylie et al., 2014 showed that loss of p53 activity does not impact DNA DSB repair kinetics in irradiated adult Drosophila ovary and it proceeds occurs at a similar rate as controls (Wylie et al., 2014). Similarly cultured mouse embryonic fibroblasts lacking p53 do not show alterations in the presence or number of γH2AX foci in response to chemically induced DNA damage compared to wild-type cells (reviewed in Reinhardt and Schumacher, 2012). In order to understand the impact of p53 on DNA DSB

repair in Drosophila testis, we immuno-stained $p53^{-/-}$ and $p53^{-/+}$ testes with anti-sera raised against DNA DSB marker γH2AvD and Zinc finger homeodomain 1 (Zfh1), a marker for CySC and immediate daughters (Leatherman and Dinardo, 2008) at different time points post exposure to 75 Gy (Supp. Fig. S3.3). GSCs were identified as non Zfh-1 positive cells located next to hub. Both $p53^{-/-}$ and $p53^{-/+}$ testes show robust and equivalent levels of γH2AvD foci in all cells of the testis apex within one hour of radiation exposure (Supp. Fig. S3.3A, B). By one day after radiation exposure, γH2AvD foci were diminished in the hub and the CySCs but not GSCs of $p53^{-/+}$ testes (Supp. Fig. S3.3C, D). Compared to irradiated heterozygous controls, hub cells and GSCs retain high levels of γH2AvD foci in testes lacking p53 (Supp. Fig. S3.3C, D) whereas, the CySCs have diminished levels of γH2AvD foci similar to heterozygous controls (Supp. Fig. S3.3C, D, white arrows). This data suggests that p53 plays a major role in promoting DNA damage repair in the hub and the GSCs but not in the CySCs. Additionally, our data shows that unlike CySCs and the hub, the GSCs (Supp. Fig. S3.3C, D white arrowheads), retain high γH2AvD levels in both $p53^{-/+}$ and $p53^{-/-}$ testes one day after radiation exposure indicating that GSCs repair their DNA DSBs at a rate different from CySCs and the hub. As mentioned previously, quiescent cells are known to use NHEJ to repair DNA damage (reviewed in Cheung and Rando, 2013). Since hub cells are known to be quiescent, it is likely that they use the NHEJ repair pathway to mend DNA damage. It is possible that like the hub, CySCs also use NHEJ to repair their DNA, while GSCs use HR to repair their DNA and thus retain unrepaired DNA DSBs even when the hub and the CySCs have repaired theirs.

As shown previously, most GSCs are lost in a week post IR exposure in $p53^{-/-}$ testes (Fig. 3.4I-M, O). We wondered if this loss of GSCs occurs due to lack of DNA repair in absence of p53. If this is true, we would expect any remaining GSCs in $p53^{-/-}$ testes, one week post-IR exposure to have unrepaired DNA DSBs. Additionally, since CySCs seem to repair DNA DSBs independent of p53 activity, we would expect $p53^{-/-}$ testes to have CySCs with no un-repaired DNA DSBs. Indeed, one week after radiation exposure, we found that the remaining GSCs in $p53^{-/-}$ testes had unrepaired DNA DSBs as identified by immuno-stain for γH2AvD (Supp. Fig. S3.3E-F, white arrowheads). CySCs in these $p53^{-/-}$ testes did not show any γH2AvD immuno-stain, as expected (Supp. Fig. S3.3E-F, white arrows). Further, the hub continued to retain γH2AvD signal in $p53^{-/-}$ testes, one week post IR exposure, suggesting that p53 is required in hub to repair DNA (Supp. Fig. S3.3E-F, red outline). Contrary to $p53^{-/-}$ testes, $p53^{-/+}$ testes had no unrepaired DNA DSBs in the GSCs (Supp. Fig. S3.3E-F, white arrowheads) or CySCs (Supp. Fig. S3.3E-F, white arrows) as shown by absence of yH2AvD in these cells one week post-IR. The hub in $p53^{-/+}$ testes did retain some yH2AvD signal but at a lower intensity than the hub in $p53^{-/-}$ testes (Supp. Fig. S3.3E-F, red outline). Our data suggests that persistent unresolved DNA DSBs contribute to the long term loss of GSCs in $p53^{-/-}$ testes. Survival of hub cells with unresolved DNA DSBs could be due to the fact that they are quiescent. Altogether, our data shows that DNA DSB repair in the hub and GSCs but not CySCs is dependent on p53 activation and that the GSCs are slow to repair their DNA compared to the hub or CySCs probably because they use HR instead of NHEJ as repair pathway.

DNA damage-induced GSC regeneration requires HR but not NHEJ pathway members

Typically, DNA damage repair pathways viz. HR and NHEJ are thought to be redundant (Johnson-Schlitz et al., 2007). If cells are not able to repair DSB through one pathway, they use a different one to complete repair. In order for successful regeneration of lost GSCs by the GSCs that remain at the hub after radiation exposure, successful DNA damage repair is required. We wondered if surviving GSCs use a particular DNA DSB pathway to repair DNA DSBs. As discussed previously, based on rate of repair of DSBs (Supp. Fig. S3), it is likely that GSCs use HR to repair DSBs probably because it is less mutagenic than NHEJ. However, it is still formally possible that GSCs use a slower version of NHEJ. It is also possible that GSCs can use either of HR or NHEJ to repair DNA damage depending on availability of the pathway. In order to distinguish between these possibilities, we examined testes from flies lacking proteins essential for the HR or NHEJ pathways. If the inability to use a specific pathway led to increased loss of GSCs post-IR, it would suggest that the particular pathway is needed for GSCs to regenerate post-IR. In contrast, if DNA repair pathways are redundant in GSCs, we would not expect the absence of a particular pathway to affect this regeneration. We first tested the requirement for the NHEJ repair pathway in our DNA damage-induced regeneration model. Flies null for *lig4* lack the protein Lig4, which is essential for NHEJ in germ cells (Johnson-Schlitz et al., 2007). Additionally, hubs in testes lacking Lig4 retain higher levels of γH2AvD compared to controls one day after radiation exposure (Supp. Fig. S3.4E-F), because

they cannot perform NHEJ which is the only pathway available to hub cells owing to their quiescence (reviewed in Cheung and Rando, 2013). Before IR, *lig4*$^{-/Y}$ testes were phenotypically undistinguishable from wild-type testes, indicating that, as expected, Lig4 is not required for GSC maintenance or activity under physiological conditions (Supp. Fig. S3.4A-D). Post-IR, testes from *lig4*$^{-/Y}$ also pheno-copied those from wild-type flies: half of the GSCs initially were depleted from the niche, and the niche was replenished within a week post-IR (Supp. Fig. S3.4A-D). This result suggests that NHEJ is not required for GSC maintenance or regeneration upon IR in the Drosophila testis.

Using a similar approach, we next tested the requirements for the HR pathway by removing function of the *blm,* gene. Blm is required in adult Drosophila testis germ cells to repair DNA DSBs using HR (Johnson-Schlitz and Engels, 2006). There was no significant difference in GSC number in heterozygous (*blm*$^{-/+}$) controls (mean= 5 GSCs/testis, N=12 testes) and blm$^{-/-}$ flies (mean= 2.7 GSCs/testis, N=11 testes) one day after exposure to 75 Gy IR (Fig. 3.6A-D, H). However, while GSC numbers returned to normal levels after one week of recovery from IR in control *blm*$^{-/+}$ flies (mean= 8.16 GSCs/testis, N=18 testes), all GSCs were lost in *blm*$^{-/-}$ flies (N=23 testes, $p<0.001$) (Fig. 3.6E-F, H). This result suggested that HR pathway is essential for regeneration of GSCs after initial loss post-IR.

To further test the requirement for HR pathway members in GSC regeneration post-IR, we next to examined flies lacking the Rad51 protein, encoded by the Drosophila *spindle*-A *(spnA)* gene. Rad51 is required for germ cells to complete DNA DSB repair by HR in adult Drosophila testes (Johnson-Schlitz et al., 2007). Similar to

our observation with *blm*, we observed no significant difference in GSC number between *spnA*$^{-/-}$ and *spnA*$^{-/+}$ flies one day after exposure to 75 Gy IR (Fig. 3.6G-J, M). However, one week after exposure to radiation, GSC number was significantly reduced in flies lacking Rad51 (*spnA*$^{-/-}$) to an average of 3.07 GSCs/ testis (N=39 testes) compared to an average of 9.2 GSCs/testis (N=20 testes) in heterozygous controls (*spnA*$^{-/+}$) ($p<0.0001$) (Fig. 3.6K-L, N). These observations further support the idea that HR pathway members play a non-redundant role in successful regeneration of GSCs after IR-induced loss.

We next wondered if there is a cell-autonomous requirement for HR but not NHEJ pathway members in GSCs regenerate post-IR. In order to test this requirement, we used the Gal4-UAS system to knockdown NHEJ and HR pathway members specifically in GSCs. Using Nanos-Gal4, we over-expressed RNAi against Ku70 (which is a protein required for NHEJ to occur (Johnson-Schlitz et al., 2007) in GSCs and their immediate daughters (Fig. 3.7D, H, L). Similar to flies completely lacking (*lig4*$^{-/Y}$) (Supp. Fig. S3.3), overexpression of Ku70 RNAi in GSCs did not significantly reduce their numbers (average of 5.08 GSCs/testis, N=24 testes) compared to controls (average of 5.72 GSCs/testis, N=18 testes) one day after exposure to radiation (Fig. 3.7M). No significant difference was observed in the number of GSCs in testes overexpressing Ku70 RNAi one week after exposure to radiation (average of 8.19 GSCs/testis, N=22 testes) compared to controls (average of 6.77 GSCs/testis, N=32 testes) (Fig. 3.7N). This result further supports the hypothesis that NHEJ pathway members are not required for regeneration of GSCs post radiation

exposure, suggesting that NHEJ repair is not the major pathway used by damaged adult Drosophila GSCs.

Using a similar approach to those described above, we tested the cell-autonomous requirement for the HR pathway member Rad51 in GSCs by knocking down Rad51 in GSCs and their immediate daughters using Rad51 RNAi driven by Nanos-Gal4. We used two different Rad51 RNAi lines represented as Rad51 RNAi (I) and Rad51 RNAi (II). Since male flies completely lacking Rad51 do not show any development abnormality with respect to the adult testis (Fig. 3.6G, H, N), we did not use $Gal80^{ts}$ to temporally regulate the expression Rad51 RNAi. Interestingly, we noticed significantly fewer GSCs (average of 8.47 GSCs/testis, N=19 testes) in un-irradiated testes expressing Rad51 RNAi (II) compared to an average of 10.27 GSCs/ testis (N=18 testes) in un-irradiated controls ($p<0.05$) (Fig. 3.7A, C, Supp. Fig. S3.2 B), suggesting that Rad51 RNAi (II) may have some off-target effects. No such reduction in GSC counts was observed in un-irradiated testes over-expressing Rad51 RNAi (I) (average of 9.8 GSCs/testis, N=20 testes) compared to controls (10.27 GSCs/ testis, N=18 testes) (Fig. 3.7A, D, Supp. Fig. S3.2B). Similar to flies completely lacking Rad51 ($spnA^{-/-}$), overexpression of Rad51 RNAi (I) in GSCs did not significantly reduce their numbers (average of 4.04 GSCs/testis, N=22 testes) compared to controls (average of 5.72 GSCs/testis, N=18 testes) one day after exposure to radiation (Fig. 3.7A-B, E-F, M). Interestingly, over-expression of Rad51 RNAi (II) showed extreme reduction of GSC numbers at one day (average of 2.76 GSCs/testis, N=25) post IR exposure compared to controls (average of 5.72 GSCs/testis, N=18 testes, $p<0.0001$) (Fig. 3.7C, G, M). Severe loss in GSC numbers

observed in testes over-expressing Rad51 RNAi (II) one day after IR was not observed in flies completely null for Rad51, further suggesting that Rad51 RNAi (II) may have off-target effects. Therefore, we only used observations with Rad51 RNAi (I), to conclude that Rad51 does not play a role in GSC loss one day post IR.

We next wondered if Rad51 plays a cell-autonomous role in regeneration of lost GSCs from the GSCs that survived one day post IR. Similar to *spnA*$^{-/-}$ flies, significant reduction was observed in GSC counts in testes with germline-specific Rad51 RNAi (I) knockdown one week after exposure to radiation (average of 4.92 GSCs/testis, N=28 testes) compared to controls (average of 6.77 GSCs/testis, N=32 testes, $p<0.05$) (Fig. 3.7I-J, N). Furthermore, one week after IR, GSC count in testes over expressing Rad51 RNAi (II) fell significantly to an average of 1.3 GSCs/testis (N=25) compared to controls (average of 6.77 GSCs/testis, N=32 testes, $p<0.0001$) (Fig. 3.7K, N). Thus, GSCs autonomously require Rad51 to recover from 75 Gy IR. Taken together the above data suggest that GSCs impaired in using HR but not NHEJ are unable to replenish the lost GSCs post-IR compared to wild-type controls. Our results highlight the role of HR in GSC regeneration post-IR induced GSC loss in Drosophila testis and strongly suggest that unlike most cells, which can use these two pathways redundantly, adult male GSCs preferentially use HR, and not NHEJ to repair IR-induced DNA DSBs in Drosophila.

Altogether, this study supports a model where within a day, testes in irradiated adult male Drosophila lose GSCs in a Chk2 and p53-dependent manner. One day after radiation exposure, the surviving GSCs either complete DNA DSB repair, detach from the niche and most likely die if the DNA damage is too severe (as in

testes exposed to 100 Gy). If DNA DSBs are to be repaired, members of the HR pathway are used preferentially over those of the NHEJ members to fix damaged DNA. Within a week post-IR exposure, the GSCs repair DNA damage, divide and replenish the lost GSCs.

Discussion

DNA damage induces Chk2 and p53 dependent loss and regeneration of GSCs

Activation of Chk2 kinase, which regulates DNA damage repair, cell cycle arrest, and apoptosis, is a highly conserved response to radiation in all model organisms including mice, Drosophila, yeast, and worms (reviewed in Zannini et al., 2014). In Drosophila, Chk2 activity has been well-characterized in the embryo (Masrouha et al., 2003), larval wing disc (Wells and Johnston, 2012), and adult ovary (Ma et al., 2016). In this study, we highlight the role of Chk2 in adult Drosophila testes, where we observed Chk2-dependent loss of GSCs in response to radiation. A similar loss of cells has been observed in many other systems such as the adult mouse thymus, where thymocytes undergo Chk2-dependent apoptosis in response to radiation (reviewed in Zannini et al., 2014) . Likewise, Chk2 mediates loss of GSCs in response to radiation in the adult Drosophila ovary (Ma et al., 2016).

Chk2 is thought to mediate apoptosis in response to radiation via p53 in Drosophila larval wing discs (Xu et al., 2001) and embryos (Brodsky et al., 2004); however, Ma et al., 2016 proposed a different mechanism for Chk2-mediated loss of GSCs in irradiated adult ovaries. They showed that Chk2 represses the expression of the germline differentiation factor *bag of marbles (bam)* in cystoblasts after the adult female flies are exposed to radiation (Ma et al., 2016). This repression of *bam* causes improper differentiation of germ cells, which accumulate in the ovary as cystoblast-like cells (Ma et al., 2016). We observed a similar IR-induced detachment of GSCs in adult males, resulting in accumulation of single germ cells in the differentiating cell zone of the testis. It is not clear if *bam* repression via Chk2 plays a role in testis.

Unlike Ma et al., 2016, however, we observed a complete recovery of GSCs and differentiating germ cells within a week after radiation; therefore, if Chk2 plays any role in repressing *bam* in the testis, the effect is temporary.

In the current study, we found that loss of p53, a known downstream effector of Chk2 kinase, completely pheno-copies the Chk2 null phenotype in the Drosophila testis under irradiation conditions. Activation of p53 can lead to cell cycle arrest and DNA damage repair; it can also lead to apoptosis if the DNA damage is too severe to be repaired (reviewed in Menon and Povirk, 2014). Here, we show that p53 activation mediates a two-step response to radiation exposure in GSCs in the adult Drosophila testis. The first step is an acute response where some GSCs are lost in a Chk2 and p53-dependent manner immediately after radiation exposure. A similar response has been seen in the developing central nervous system in mice (Herzog et al., 1998) and in the Drosophila embryo, where p53 activation induces cell death (Brodsky et al., 2004). In addition to the acute response, we also observe a second response that occurs in the GSCs that remain at the niche one day after IR exposure. In this second response, a week after radiation exposure: GSCs that lack p53 are gradually lost, while those that remain are able to regenerate lost GSCs in a p53-dependent manner. Interestingly, Wylie et al., 2014 and Ma et al., 2016 observed complete loss of fertility in p53 null adult ovaries within a week after DNA damage induced by radiation (115 Gy) (Wylie et al., 2014) or endonuclease activity (I-CreI) (Ma et al., 2016). Additionally, we observed that like GSCs lacking p53, GSCs deficient in proteins required for HR repair are gradually lost after IR exposure. Therefore, our data suggest that p53 mediated activation of HR repair in GSCs that remain at the hub

one day after IR exposure, leads to regeneration of lost GSCs in a week after irradiation.

GSCs in the adult Drosophila testis prefer HR over NHEJ to repair DNA DSBs

DNA DSBs can be repaired by either HR or NHEJ. Since HR is less mutagenic than NHEJ, one would imagine that HR would be the preferred mode of DNA repair in GSCs, to prevent transmission of mutations across generations through the germline. Whether or not a particular DNA double strand break repair pathway is preferred in GSCs has not been studied *in vivo* in any system. The adult Drosophila testis is a well understood system that is ideal for addressing this question. Work by Preston et al., 2006 showed that young Drosophila males repair DNA in germ cells by NHEJ while older males use HR. However, the promoter used in this study to induce DNA double strand breaks is expressed in spermatocytes but not in GSCs; hence the results not apply to GSCs (Preston et al., 2006). Here, we show that GSCs in the adult Drosophila testis require HR but not NHEJ pathway members to regenerate GSCs that are lost in response to IR. Our results contradict previous studies in Drosophila testis germ cells, which suggested that DNA double strand repair pathways are inter-changeable and that if one pathway is blocked, the other can be used instead (Johnson-Schlitz et al., 2007). By contrast, our results suggest that HR and NHEJ pathways are not interchangeable in Drosophila testis GSCs and that GSCs lacking HR are lost because their damaged DNA cannot be repaired. This conclusion is supported by the fact that HR pathway proteins have no known function besides DNA damage repair in testis GSCs. Studies using reporters to directly assay DNA damage repair in GSCs are needed to confirm this hypothesis.

The mechanisms that ensure repair by HR in GSCs are not yet clear. One possible mechanism is transcriptional or translational regulation of specific repair pathway members in GSCs. Interestingly, publically available gene expression data show an enrichment of Rad51 and Blm proteins compared to Lig4 in GSC-like cells from the testis (Terry et al., 2006). This enrichment could be actively maintained through niche signals. Alternatively, GSCs could intrinsically express HR pathway members at higher levels than NHEJ members.

Materials and Methods

Fly Stock and Culture

Flies were raised on standard molasses media with sprinkling of dry yeast at 25 º C. The following stocks were used:

UASp p35-FLAG (kind gift from Dr. T. Xie) (Ma et al., 2016), *mnk*^{p6} (kind gift from Dr. Z. Zhang) (Ma et al., 2016), *Nanos-Gal4* (kind gift of Dr. E. Selva) (White-Cooper, 2012), *SpnA*^{093}*/TM3* (kind gift from Dr. J. LaRocque) (Delabaere et al., 2016), *HS-FLP/Y; Tubulin > STOP > Gal4, UAS GFP* (kind gift from Dr. B. Ohlstein). Other stocks were sourced from the Bloomington Drosophila Stock Center (BDSC). The following BDSC RNAi lines were used *Rad51 RNAi (I)* (BDSC-51926), *Rad51 RNAi (II)* (BDSC-38898), *Chk2 RNAi* (BDSC-35152) and *Ku70 RNAi* (BDSC-61942). *UAS p53*^{R155H} (BDSC-8419) was used.

Transgene expression in GSCs

RNAi expression for Rad51, Ku70 and Chk2 as well as over expression of p53^{DN} was achieved by setting up the cross between virgin Nanos Gal4 females and males containing the *UAS RNAi* or *UAS p53*^{DN} transgene. The cross was set at 29 ºC. Adult male progeny were collected and kept at 29 ºC for the indicated amount of time before their testes were dissected and fixed. Crosses for expression of Chk2 RNAi and p53^{DN} were set up at the same time and in the same 29 ºC incubator. We pooled GSC counts from sibling controls of both crosses (UAS Chk2 RNAi crossed to Nanos Gal4 and UAS p53^{DN} crossed to Nanos Gal4) at each time-point because no significant difference was found between GSC counts from sibling controls at any

time-point. Similarly, GSC counts were pooled for the sibling controls for Rad51 RNAi (both I and II) as well as Ku70 RNAi.

Detection of Apoptosis

Staining for apoptosis was done using the Millipore Apoptag kit S7160 as per the manufacturer's instruction and the protocol outlined in (Hasan et al., 2015).

Quantification of dead cells

The dead cells were quantified by counting the TUNEL positive spots in the control as well as the irradiated testes. TUNEL positive spots adjacent to the hub were identified as dead GSCs, while those more than 1 nuclei diameter away from the hub were identified as dead differentiating germ cells.

Mosaic Analysis

Newly ecclosed males of the genotype *HS-FLP/Y; Tubulin > STOP > Gal4, UAS GFP* were heat shocked 3 times for 40 minutes at 37 °C with intervals of 30 minutes at 25 °C to induce clones. The flies were then maintained at 29 °C for 2 days after clone induction, then irradiated at 75 Gy. After radiation, flies were maintained at 29 °C after radiation exposure and dissected 1, 2 and 3 days after radiation exposure. Clones were counted as described in the text.

Testis immunocytochemistry

Testes were dissected, fixed and immuno-stained as per the protocol in (Matunis et al., 1997) The following primary antibodies were used rabbit anti-Vasa at 1:200 (Santa Cruz Biotechnology), mouse anti-Hts (1B1) at 1:50 (Developmental studies hybridoma bank), guinea pig anti-Zfh-1 at 1:500, chicken anti-GFP at 1:10000 (Abcam), mouse anti-FLAG at 1:500 (Sigma life sciences) and rabbit anti-γH2AvD at 1:1000 (Rockland Biosciences). Nuclei were counterstained with 1 μg/ml 4′-6-diamidino-2-phenylindole (DAPI) (Roche Molecular Biochemical). Stained testes were mounted in vectashield and imaged.

Ionizing radiation

Young (0-4 days old) adult male flies were irradiated at 50 Gy, 75 Gy or 100 Gy with γ rays. Gammacell 3000 Elan with Cesium 137 as the radiation source was used to irradiate the flies.

Live imaging

Same-aged irradiated and control males were dissected immediately after radiation exposure. Testes were isolated, cultured and live-imaged as described in Greenspan and Matunis, 2017 on Zeiss LSM 780 confocal microscope.

Image acquisition

Zeiss LSM 5 PASCAL microscope or Zeiss LSM 700 microscope was used to acquire confocal in the linear range for intensity for all channels

Statistical analysis

All statistical tests were performed using Prism 6 (GraphPad Software).

Author Contributions

S.H. and E.M. designed the experiments. S.H. performed experiments and data analysis. S.H. and E.M. wrote the manuscript.

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Table legends

Table 3.1. Wild-type single germ cell clones are found away from hub in irradiated testes

Testes with one or more clones have been quantified both as a fraction of total number of testes and as percentages (in brackets). P-values are based on comparisons between the percent testes with wild-type clones of indicated type (GSC, CySCs, spermatogonia, cyst cells or single germ cells away from hub) in un-irradiated and irradiated testes. HS= Heat shock, ACI= After clone induction. P-values based on 2-tailed, Fisher's exact test.

Figure legends

Figure 3.1. Regeneration of Germline stem cells (GSCs) in the testis niche occurs at a specific radiation dose of 75 Gy.

(A) Illustration of the Drosophila adult testis niche. GSCs (yellow) and Cyst stem cells (CySCs) (dark blue) are adjacent to the hub at the testis apex. GSCs divide to give rise to gonialblasts, which continue to divide, forming spermatogonial cysts. CySCs divide to form cyst cells, which envelop gonialblasts and their progeny. GSCs were counted under different radiation conditions, as germ cells adjacent to the hub containing a round fusome (red).

(B-G) Confocal sections of testes immuno-stained with anti-Vasa antisera (marks germ cells, red), anti-Hts antisera (fusome, green) and DAPI (DNA, blue) under control conditions (B), 1 day after exposure to 50 Gy γ radiation (C), 1 day after exposure to 75 Gy γ radiation (D), 7 days after exposure to 75 Gy γ radiation (E), 1 day after exposure to 100 Gy γ radiation (F) and 7 days after exposure to 100 Gy γ radiation (G). Hub is outlined in white, Scale bar 10 μm.

(H) Scatter plots showing the number of GSCs per testis apex. Upon 50 Gy of irradiation, there is no significant difference in number of GSCs compared to un-irradiated controls. In contrast, 75 Gy of IR causes significant reduction in GSC count in a day compared to un-irradiated controls. However the GSC count recovers to that of un-irradiated controls in a week. Similarly, 100 Gy of IR causes significant reduction in GSC count in a day compared to un-irradiated controls. However the remaining GSC count never recovers and most of the testis lose all germ cells in a

week post 100 Gy IR. ** p<0.01, *** p<0.001, ****p<0.0001, 2-tailed Kruskal-Wallis test with multiple comparisons. Bars indicate mean and S.D.

Figure 3.2. Apoptosis is not responsible for loss of GSCs from the niche post 75 Gy radiation.

(A) Confocal sections of testis immuno-stained with TUNEL (apoptotic cells, red) and DAPI (DNA, blue) under control conditions (A) and 3 hours after exposure to 75 Gy γ radiation (B). TUNEL-positive cells were quantified in a "GSC zone" (bounded by the hub and yellow dashed line) that comprised of the first tier of cells adjacent to the hub which includes all GSCs. TUNEL-positive spots (arrows) are not seen next to the hub in both control and irradiated testis.

(C) Scatter plot showing the number of TUNEL-positive spots per testis apex in the GSC zone. Upon 75 Gy of radiation, there is no significant difference in TUNEL-positive spots in GSC zone compared to un-irradiated controls. 2-tailed Mann-Whitney U test. Bars indicate mean and S.D.

(D-G) Confocal sections through the apex of testes immuno-stained with anti-Vasa antisera (marks germ cells, red), anti-Hts antisera (fusome, green) and DAPI (DNA, blue). Control testes and testes expressing caspase-inhibitor p35 in GSCs and their immediate daughter using Nanos-Gal4 were subjected to control conditions (D, E) or exposure to 75 Gy radiation (F, G). Irradiated testes show loss of germ cells compared to un-irradiated controls.

(H) Scatter plots showing the number of GSCs per testis apex in un-irradiated testes and testes exposed to 75 Gy radiation. Number of GSCs per testis apex is significantly higher in un-irradiated testes over-expressing p35 in GSCs and immediate daughters compared to un-irradiated controls. Significant loss of GSCs occurs post-radiation in both control and testes over-expressing p35 in GSCs and daughters. **** $p<0.0001$, 2-tailed ordinary one way ANOVA test with multiple comparisons. Bars indicate mean and S.D.

(I-J) Confocal sections through the apex of testes immuno-stained with anti-FLAG (white, shows p35 expression), anti-Vasa (A-C, germ cells, red) and DAPI. (I'-J') FLAG channel alone. Testes were imaged at the same gain to show different levels of p35-FLAG protein in (I, I') a control testis or in testes overexpressing p35-FLAG in (J, J') GSCs and their daughters using Nanos Gal4. Hub is outlined in white, Scale bar 10 μm.

Figure 3.3. GSCs leave the niche instead of dying there post 75 Gy radiation.

(A-B) Confocal sections through the testis apex show un-irradiated (A,A') or irradiated wild-type clones 1 day after exposure to 75Gy radiation (B,B'). To quantify single germ cells away from hub, testes were divided into 1) "stem cell zone" comprising of the first two tiers of cells adjacent to the hub which includes GSCs and gonialblasts which are the only single germ cells in testis apex 2) "differentiating germ cell zone", which extends beyond the stem cell zone. This zone includes interconnected spermatogonial clusters but usually no single germ cell. The yellow dashed line separates the two zones in the testis apex.

Germ cell clones are identified by the presence of GFP (green) and vasa (red) immuno-stains. Germ cell clones adjacent to hub can be identified as GSCs (dashed white arrow), spermatogonial clones are away from hub (solid white arrow). In addition to GSCs and spermatogonia, single germ cells (white arrowhead) are visible in the differentiating cell zone in irradiated testes (B) only and not in un-irradiated controls (A).

(C) Percentage of testes containing single germ cell clones are plotted as line graphs. Un-irradiated controls are shown in and irradiated testes in orange. No single germ cell was visible away from the hub in un-irradiated controls but significantly increased in number after exposure to radiation. No significant difference was seen between irradiated and un-irradiated controls in terms of percentage testes with single germ cell clones 2 days after radiation treatment. * $p<0.05$, 2-tailed Fisher's exact test. Sample size is indicated in the figure.

(E-G) Confocal sections through the testis apex imaged live after exposure to IR in an ex-vivo culture of testes. Nuclei are marked in red by transgenic expression of fusion protein Histone 2A – red fluorescent protein (His2A-RFP). GSCs and their immediate daughters are marked in green by Nanos promoter- dependent expression of GFP-actin binding subunit of moesin fused with GFP (Nos-GMA). The GSC detaching from the hub is outlined in white dotted line. The GSC detaches between the first (E) and the second frame (F). Time stamps are indicated at the bottom of each frame in minutes. Each confocal Z-series is captured at an interval of 22 minutes. (E) is the first time-point of the live-imaged movie. Hub is marked with an asterisk. Scale bar 10 μm.

Figure. 3.4. **DNA damage-induced GSC regeneration requires Chk2-mediated activation of p53.**

Fig. 4. DNA damage-induced GSC regeneration requires Chk2 mediated activation of p53

(A -M) Confocal sections through the apex of testes immuno-stained with anti-Vasa antisera (marks germ cells, red), anti-Hts antisera (fusome, green) and DAPI (DNA, blue). Hub is outline in white. Control testes and testes from flies lacking Chk2 (B, D, F) or p53 (I, K, M) were subjected to control conditions (A-B, H-I) or exposure to 75Gy radiation (C-F. J-M). Irradiated testes were stained 1 day (C-D, J-K) or 7 days (E-F,L-M) after exposure to radiation. Control testes show greater loss of GSCs compared to testes lacking Chk2 or p53, one day after exposure to 75Gy (compare A-B to C-D and H-I to J-K). GSC count is restored in control testes, 7 days after radiation exposure (E, L) whereas at this time point GSC number is reduced in testes lacking Chk2 or p53 (H,M). Scale bar 10 μm.

(G, N) Scatter plots showing quantification of GSCs in irradiated controls or irradiated testes lacking Chk2 or p53. Significantly fewer GCSs are observed in irradiated controls one day after exposure to 75Gy compared to Chk2 (G) or p53 lacking testes (N). While irradiated controls recover their GSC count in 7 days after exposure to 75Gy (G, N), GSCs are significantly reduced in number in testes lacking Chk2 (G) or p53 (N) 7 days post radiation exposure.

* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, 2-tailed One way ANOVA with multiple comparisons (G) or 2-tailed Kruskal-Wallis test with multiple comparisons

(N). In all graphs, bars indicate mean and S.D.; sample size for each treatment set indicated under the respective plot.

Figure 3.5. DNA damage-induced GSC loss requires Chk2 and p53 cell-autonomously.

(A -I) Confocal sections through the apex of testes immuno-stained with anti-Vasa antisera (marks germ cells, red), anti-Hts antisera (fusome, green) and DAPI (DNA, blue). Hub is outlined in white. Control testes and testes expressing RNAi against Chk2 (B, E, H) or dominant-negative form of p53 in GSCs and their immediate daughters using Nanos-Gal4 were subjected to control conditions (A-C) or exposure to 75 Gy radiation (D-I). Testes were stained 1 day (D-F) or 7 days (G-I) after exposure to radiation. Control testes show greater loss of GSCs compared to testes with reduced functional Chk2 or p53 in GSCs, one day after exposure to 75 Gy (compare A-C to D-F). GSC count is restored in control testes, 7 days after radiation exposure (G). GSC number is reduced in testes lacking functional Chk2 or p53 in GSCs, 7 days after radiation exposure (H-I). Scale bar 10 μm.

(J-K) Scatter plots showing quantification of GSCs in irradiated controls or irradiated testes lacking functional Chk2 or p53 in GSCs and immediate daughters. Significantly fewer GCSs are observed in irradiated controls one day after exposure to 75 Gy compared to testes expressing Chk2 RNAi or dominant negative p53 in GSCs (J). While irradiated controls recover their GSC count in 7 days after exposure to 75 Gy (K), GSCs lacking Chk2 or functional p53 (K) are significantly reduced in number compared to control. *$p<0.05$, ****$p<0.0001$, 2-tailed Unpaired Student's t-

tests. In all graphs, bars indicate mean and S.D.; sample size for each treatment set indicated under the respective plot.

Figure 3.6. **DNA damage-induced GSC regeneration requires Homologous recombination (HR) pathway members.**

(A –L) Confocal sections through the apex of testes immuno-stained with anti-Vasa antisera (marks germ cells, red), anti-Hts antisera (fusome, green) and DAPI (DNA, blue). Hub is outlined in white. Control testes and testes from flies lacking Blm (B, D, F) or Rad51 (H, J, L) were subjected to control conditions (A-B, G-H) or exposure to 75 Gy radiation (C-F, I-L). Testes were stained 1 day (C-D, I-J) or 7 days (E-F, K-L) after exposure to radiation. All testes show loss of GSCs one day after exposure to 75 Gy (compare A-B to C-D and G-H to I-J). GSC count is restored in control testes, 7 days after radiation exposure (E, K) whereas at this time point GSC number is reduced to zero in testes lacking Blm or Rad51 (H,L). Scale bar 10 μm.

(M-N) Scatter plots showing quantification of GSCs in irradiated controls or irradiated testes lacking Blm or Rad51. Number of GSCs are not significantly altered in testes lacking Blm (M) or Rad51 one day after exposure to 75 Gy compared to irradiated controls (N). While irradiated controls recover their GSC count in 7 days after exposure to 75 Gy (M-N), GSCs are significantly reduced in number in testes lacking Blm (M) or Rad51 (N). ****- $p<0.0001$, 2-tailed Kruskal-Wallis test (M) or One way ANOVA (N) with multiple comparisons. In all graphs, bars indicate mean and S.D.; sample size for each treatment set indicated under the respective plot.

Figure. 3.7 DNA damage-induced GSC regeneration requires HR pathway members cell-autonomously.

(A –L) Confocal sections through the apex of testes immuno-stained with anti-Vasa antisera (marks germ cells, red), anti-Hts antisera (fusome, green) and DAPI (DNA, blue). Hub is outlined in white. Control testes and testes expressing RNAi against Rad51 (B-C, F-G, J-K) or Ku70 (D, H, L) in GSCs and their immediate daughters using Nanos-Gal4 were subjected to control conditions (A-D) or exposure to 75 Gy radiation (E-L). Testes were stained 1 day (E-H) or 7 days (I-L) after exposure to radiation. Testes containing GSCs with reduced Rad51 (F,G) show greater loss of GSCs compared to control testes (E) or testes with reduced Ku70 (H) in GSCs, one day after exposure to 75 Gy (compare A-D to E-H). GSC count is restored in controls (I) and in testes with reduced Ku70 (L), 7 days after radiation exposure. GSC number is reduced in testes lacking Rad51 in GSCs, 7 days after radiation exposure (J-K). Scale bar 10 μm.

(M-N) Scatter plots showing quantification of GSCs in irradiated controls or irradiated testes lacking Rad51 or Ku70 in GSCs and immediate daughters. GSCs expressing Rad51 RNAi (II) are significantly reduced compared to irradiated controls one day after exposure to 75 Gy (M). At this time point, GSCs expressing Ku70 RNAi or Rad51 RNAi (I) are not significantly altered in numbers compared to controls (M). While numbers of control GSCs and GSCs lacking Ku70 recover to pre-radiation levels in 7 days after exposure to 75 Gy (N), GSCs lacking Rad51 (N) are significantly reduced.

*p<0.05, ****p<0.0001, 2-tailed Unpaired Student's t-test with equal SD. In all graphs, bars indicate mean and S.D.; sample size for each treatment set indicated under the respective plot.

Supplementary figure legends

Figure S3.1. γH2AvD foci increase with ionizing radiation dose in all cells in the testis apex.

(A-D) Confocal sections through the apex of testes immuno-stained with anti-γH2AvD (white) and DAPI. Testes were imaged at the same gain and within same time frame (1-2 hour) post-radiation exposure, to show increase in yH2AvD levels in all cells of the testes apex proportional to radiation dose. Hub is outline in red. Scale bar 10 μm.

Figure S3.2. Testes expressing RNAi against DNA damage response and repair pathway members have similar number of GSCs as controls under physiological (no-irradiation) conditions.

(A) Scatter plots showing quantification of GSCs in un-irradiated controls testes lacking functional Chk2 or p53 in GSCs and immediate daughters. GSC count in controls is similar to testes expressing Chk2 RNAi or dominant negative p53 in GSCs.

(B) Scatter plots showing quantification of GSCs in un-irradiated controls or un-irradiated testes lacking Rad51 or Ku70 in GSCs and immediate daughters. GSCs expressing Rad51 RNAi (II) are significantly reduced compared to controls whereas GSCs expressing Ku70 RNAi or Rad51 RNAi (I) are not significantly altered in number compared to controls. *$p<0.05$, Unpaired Student's t-test. In all graphs, bars

indicate mean and S.D.; sample size for each treatment set indicated under the respective plot.

Fig. S3.3. DNA double strand break repair is delayed in testes lacking p53. (A-F) Confocal sections through the apex of testes immuno-stained with antisera raised against γH2AvD (white), antisera raised against Cyst stem cells (CySC) and immediate daughter marker Zfh1 and DAPI. Testes were imaged at the same gain for γH2AvD and within same time frame (1 hour, 1 day or 7 days) post radiation exposure. 1 hour post-IR exposure, increased γH2AvD levels are visible in all cells in both $p53^{+/-}$ (A-A") and $p53^{-/-}$ testes (B-B"). By 1 day post IR, γH2AvD stain decreases in the hub and Zfh-1 positive somatic cells (white arrows) in $p53^{+/-}$ testes (C-C") but not in GSCs (some examples marked by white arrowheads) identified by lack of Zfh-1 stain and proximity to hub. At 1 day post IR, $p53^{-/-}$ testes retain increased γH2AvD levels in hub and GSCs (some examples marked by white arrowheads) but not in CySCs (white arrows) (D-D"). 7 day post IR, γH2AvD stain decreases in all cells in the testis including the hub, GSCs (white arrowheads) and Zfh-1 positive somatic cells (white arrows) in $p53^{+/-}$ testes (E-E"). However, $p53^{-/-}$ testes retain increased γH2AvD levels in hub and GSCs (some examples marked by white arrowheads) but not in CySCs (white arrows) (F-F"). Hub is outlined in red. Scale bar 10 μm.

Figure S3.4. **DNA damage-induced GSC regeneration does not require non homologous end joining (NHEJ) pathway member DNA Ligase IV (Lig4).**

(A –C) Confocal sections through the apex of testes immuno-stained with anti-Vasa antisera (marks germ cells, red), anti-Hts antisera (fusome, green) and DAPI (DNA, blue). Hub is outlined in white. Testes from flies lacking Lig4 were subjected to control conditions (A) or exposure to 75Gy radiation (B-C). Since Lig4 is on X chromosome, no heterozygous control could be used. Testes were stained 1 day (B) or 7 days (C) after exposure to radiation. Testes show loss of GSCs one day after exposure to 75 Gy (compare A to B). GSC count is restored in testes, 7 days after radiation exposure (C). Scale bar 20 μm.

(D) Scatter plots showing quantification of GSCs in irradiated testes lacking Lig4. Significantly fewer GCSs are observed in testes lacking Lig4 one day after exposure to 75 Gy. GSC numbers recover to pre-exposure levels 7 days after radiation exposure.

**p<0.01, ****p<0.0001, One way ANOVA with multiple comparisons. In all graphs, bars indicate mean and S.D.; sample size for each treatment set indicated under the respective plot.

(E-F) Confocal sections through the apex of testes immuno-stained with anti-γH2AvD (white) and DAPI. Testes were imaged at the same gain and within same time frame (1 day) post radiation exposure, to show increased yH2AvD levels in hub (red outline) cells of testes lacking lig4 (F, F') compared to wild-type (WT) controls (E,E'). Some cells next to hub (white arrows) retain high levels of yH2AvD in both WT and lig4-/Y (E-F) testes 1 day post radiation exposure suggesting these cells do not use Lig4 (NHEJ) dependent DNA repair. Scale bar 10 μm.

**p<0.01, ****p<0.0001, One way ANOVA with multiple comparisons. In all graphs, bars indicate mean and S.D.; sample size for each treatment set indicated under the respective plot.

Table

Table 3.1

Days ACI	Days after irradiation (75 Gy)	Percentage of testes with clones				
		GSC clones	CySC clones	Spermatogonial Clones	Cyst Cell Clones	Single germ cell clones >2 cell nuclei away from hub
2	Un-irradiated	36.1% (13/36)	30.1% (11/36)	66.7% (24/36)	25% (9/36)	0% (0/36)
3	Un-irradiated	23.1% (5/21)	9.5% (2/21)	80.1% (17/21)	14.3% (3/21)	0% (0/21)
	1 D	26.1% (12/46)	13% (6/46)	32.6% (15/46)	21.7% (10/46)	23% (10/46)
	p-value	ns	ns	P=0.0004	Ns	P=0.0245
5	Un-irradiated	37.5% (9/24)	16.6% (4/24)	45.8% (11/24)	16..6% (4/24)	0% (0/24)
	3 D	10.5% (2/19)	0% (0/19)	15.7% (3/19)	15.8% (0/19)	5.2%(1/19)
	p-value	ns	ns	ns	ns	ns

Figures

Figure 3.1

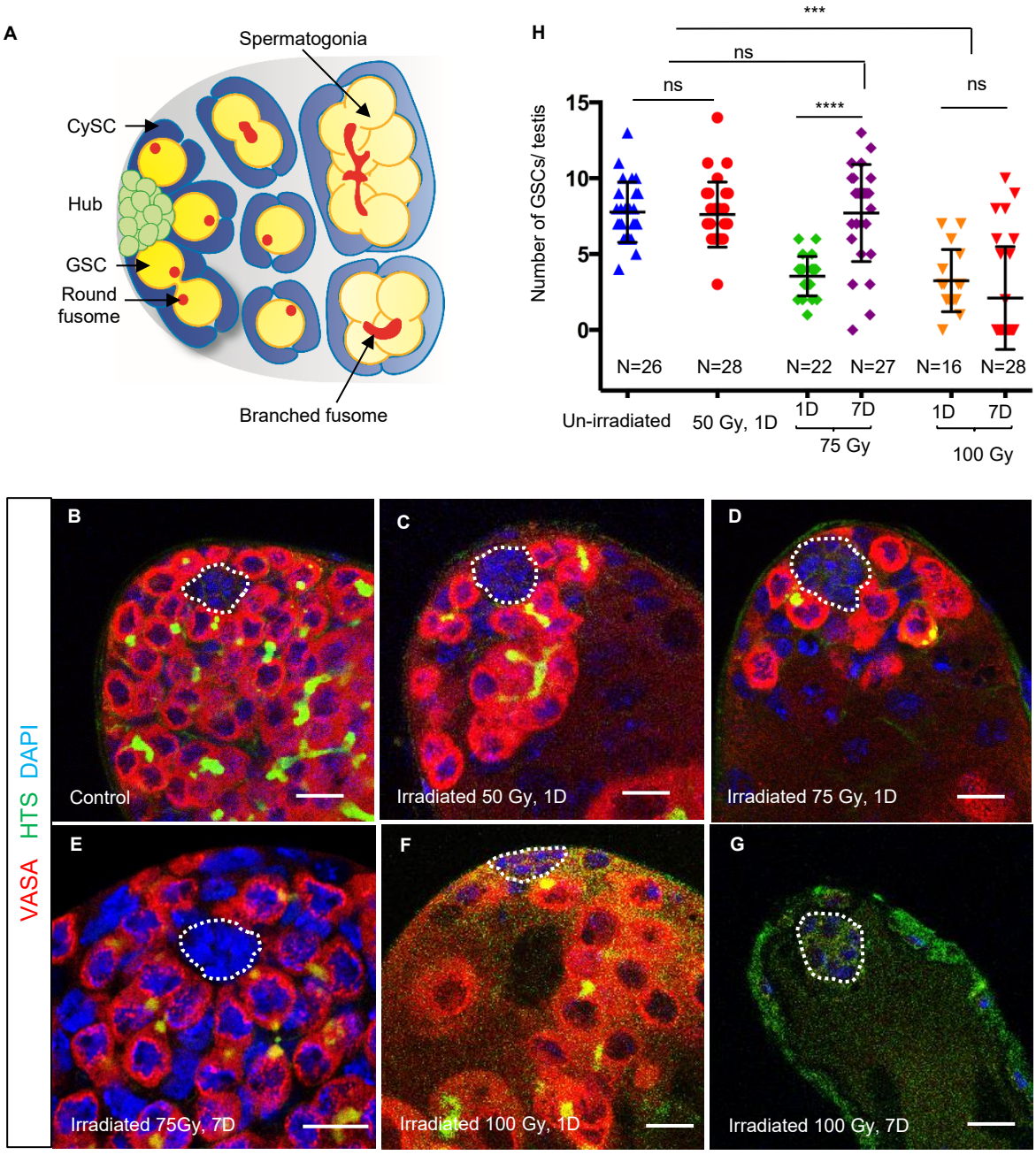


Figure 3.2

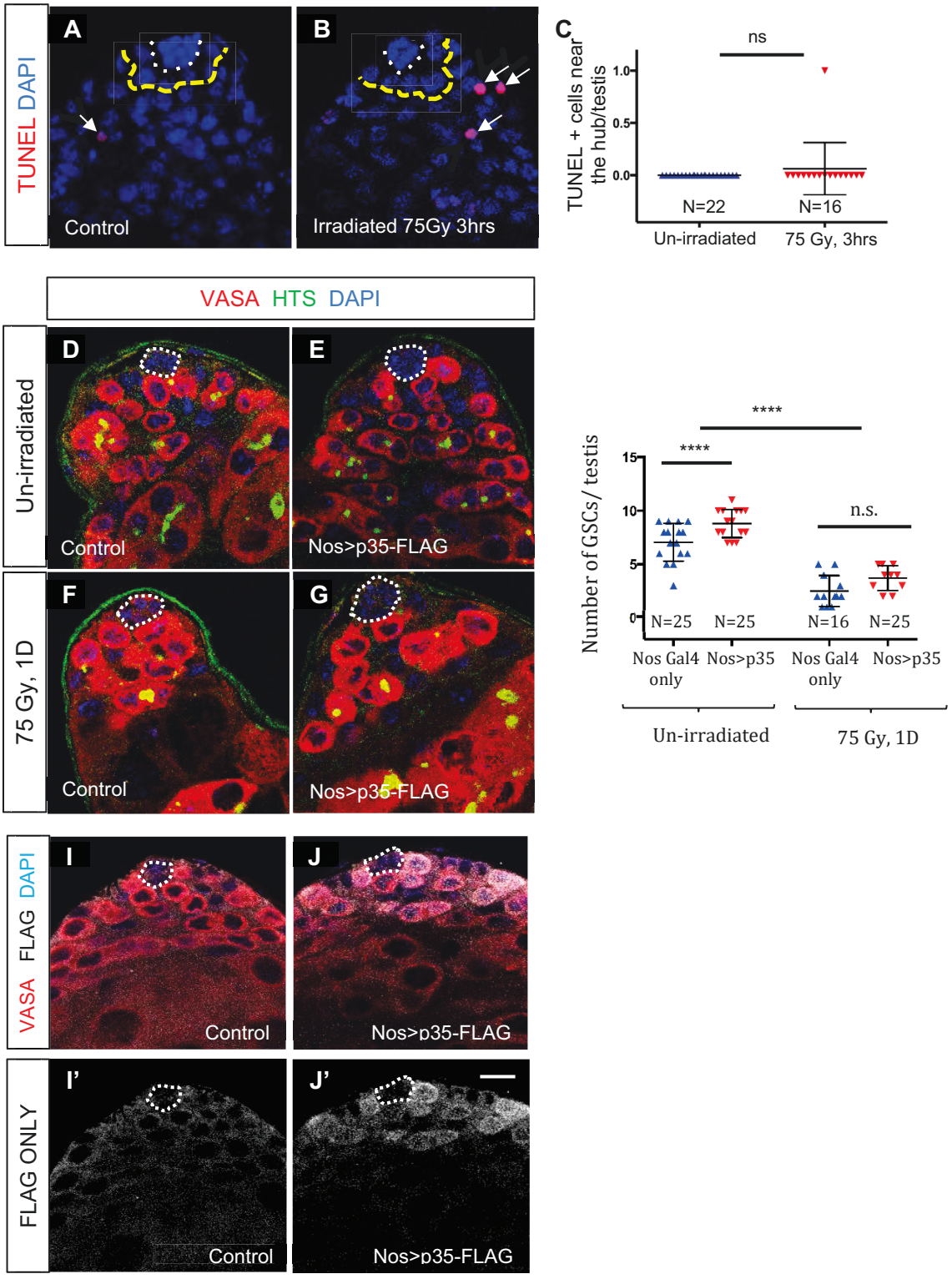

TUNEL DAPI
A
Control
B
Irradiated 75Gy 3hrs
C
ns
TUNEL + cells near the hub/testis
1.0
0.5
0.0
N=22
N=16
Un-irradiated
75 Gy, 3hrs
VASA HTS DAPI
Un-irradiated
D
Control
E
Nos>p35-FLAG
75 Gy, 1D
F
Control
G
Nos>p35-FLAG
****
****
n.s.
Number of GSCs/ testis
15
10
5
0
N=25
N=25
N=16
N=25
Nos Gal4 only
Nos>p35
Nos Gal4 only
Nos>p35
Un-irradiated
75 Gy, 1D
VASA FLAG DAPI
I
Control
J
Nos>p35-FLAG
FLAG ONLY
I'
Control
J'
Nos>p35-FLAG

Figure 3.3

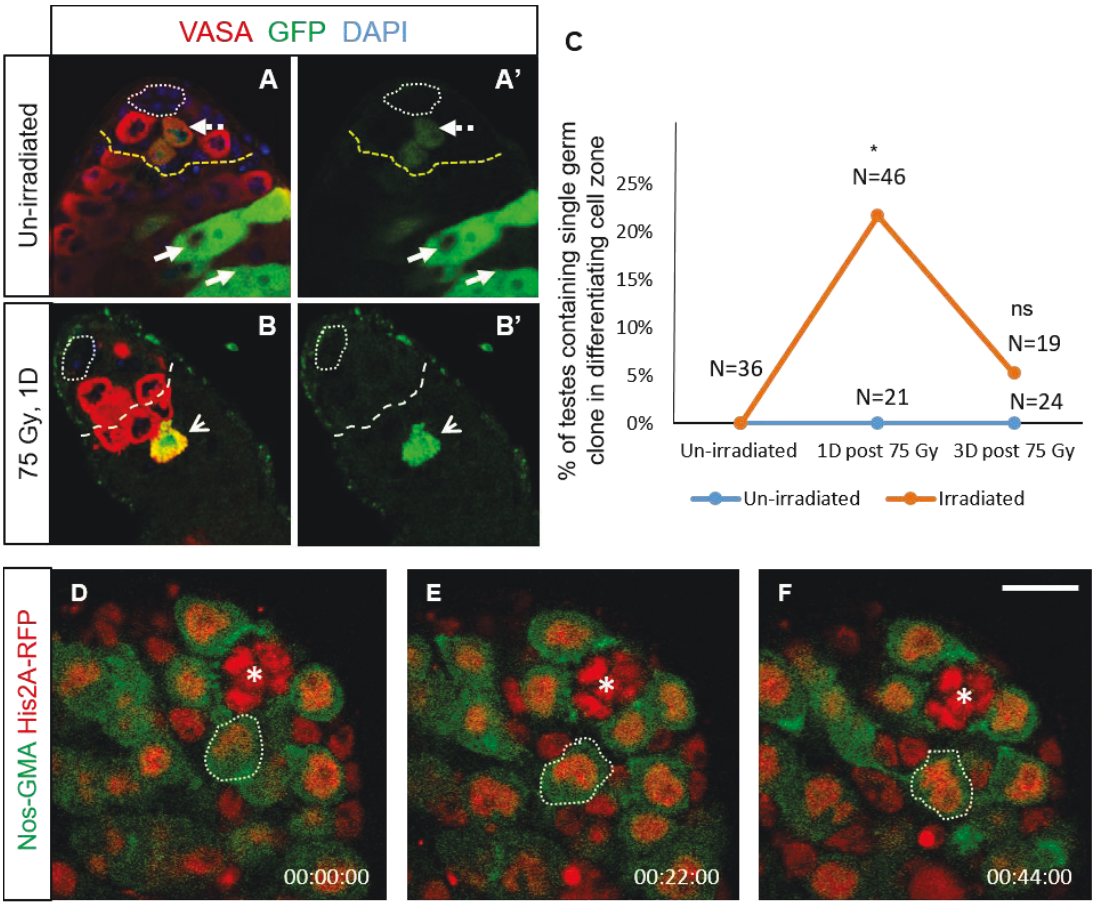

VASA GFP DAPI
Un-irradiated
75 Gy, 1D
A
A'
B
B'
C
% of testes containing single germ clone in differentiating cell zone
25%
20%
15%
10%
5%
0%
*
N=46
ns
N=19
N=36
N=21
N=24
Un-irradiated
1D post 75 Gy
3D post 75 Gy
Un-irradiated
Irradiated
Nos-GMA His2A-RFP
D
E
F
00:00:00
00:22:00
00:44:00

Figure 3.4

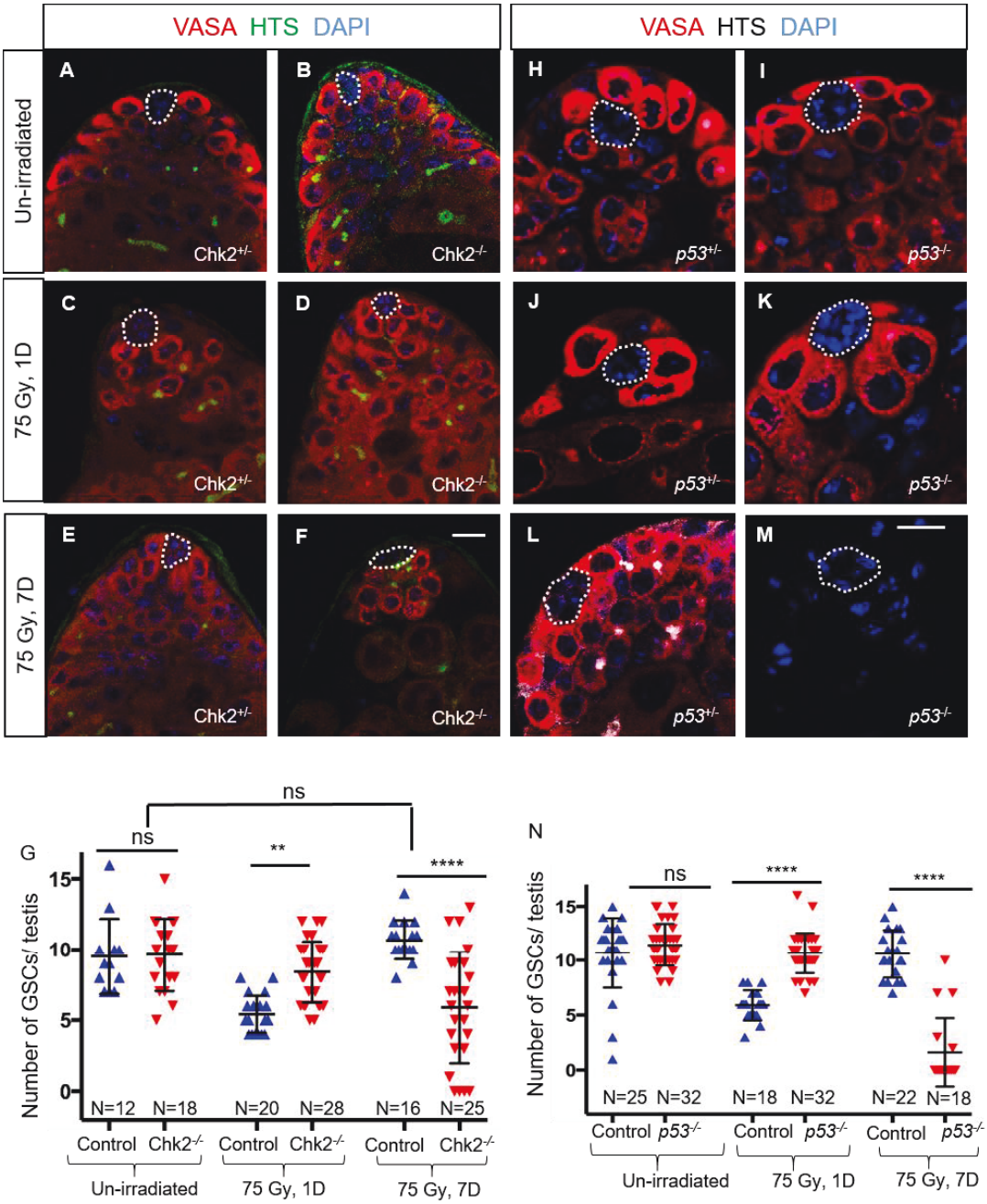

VASA HTS DAPI
VASA HTS DAPI
Un-irradiated
75 Gy, 1D
75 Gy, 7D
A
B
C
D
E
F
H
I
J
K
L
M
Chk2+/-
Chk2-/-
p53+/-
p53-/-
G
Number of GSCs/ testis
ns
**
****
N=12 N=18 N=20 N=28 N=16 N=25
Control Chk2-/-
Un-irradiated
75 Gy, 1D
75 Gy, 7D
N
N=25 N=32 N=18 N=32 N=22 N=18
Control p53-/-

Figure 3.5

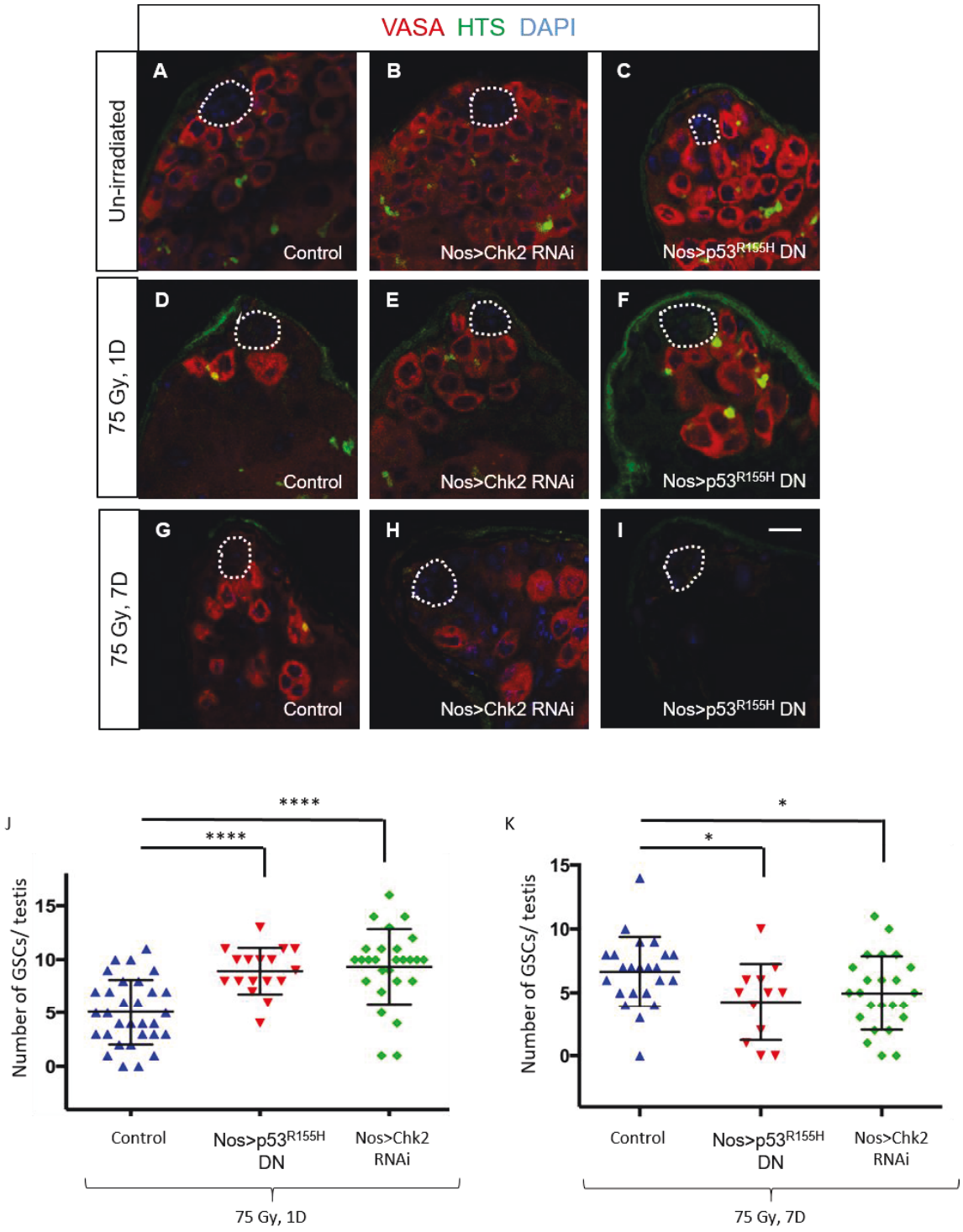

VASA HTS DAPI
Un-irradiated
75 Gy, 1D
75 Gy, 7D
A
B
C
D
E
F
G
H
I
Control
Nos>Chk2 RNAi
Nos>p53R155H DN
J
K
****
****
*
*
Number of GSCs/ testis
15
10
5
0
Control
Nos>p53R155H DN
Nos>Chk2 RNAi
75 Gy, 1D
75 Gy, 7D

Figure 3.6

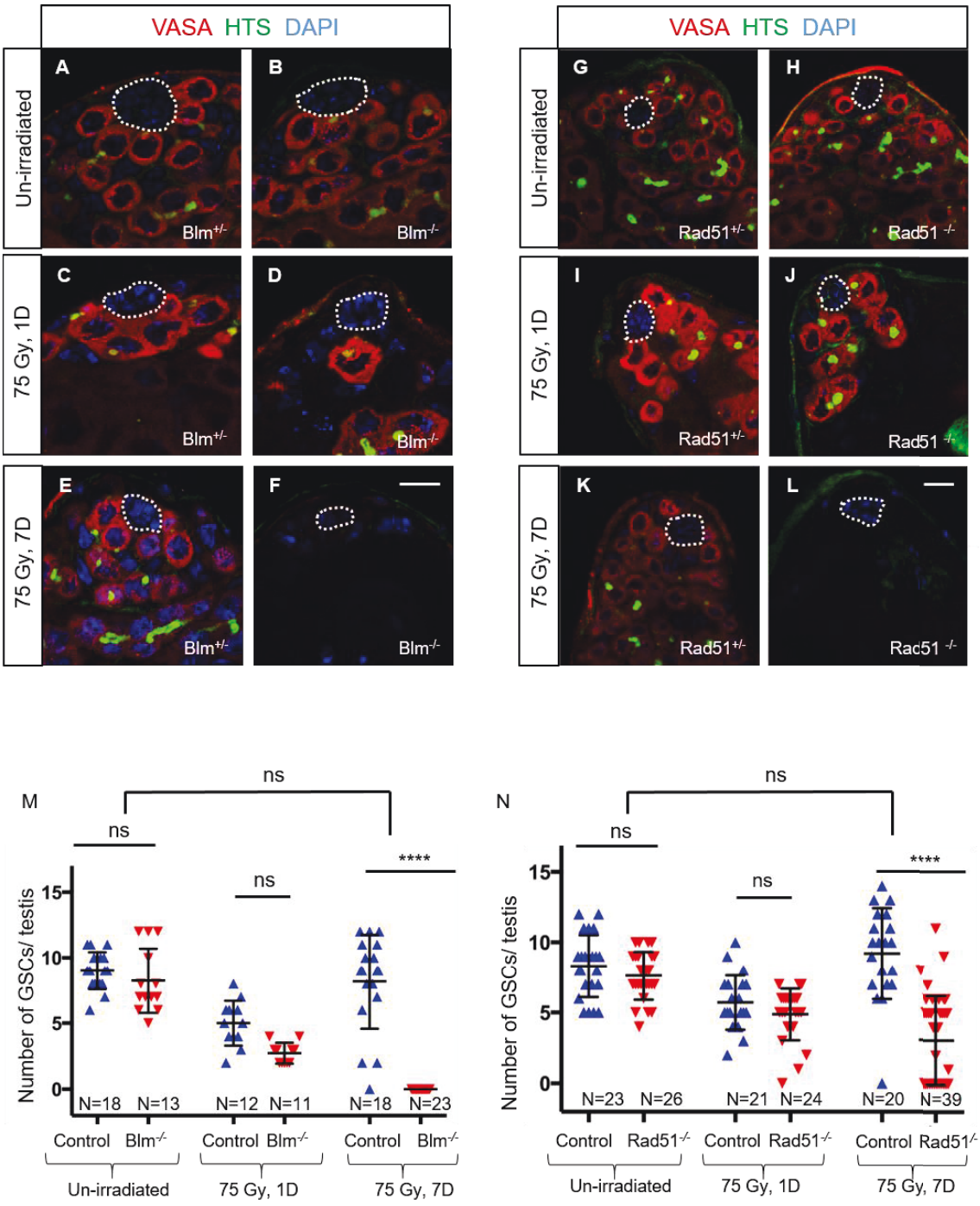
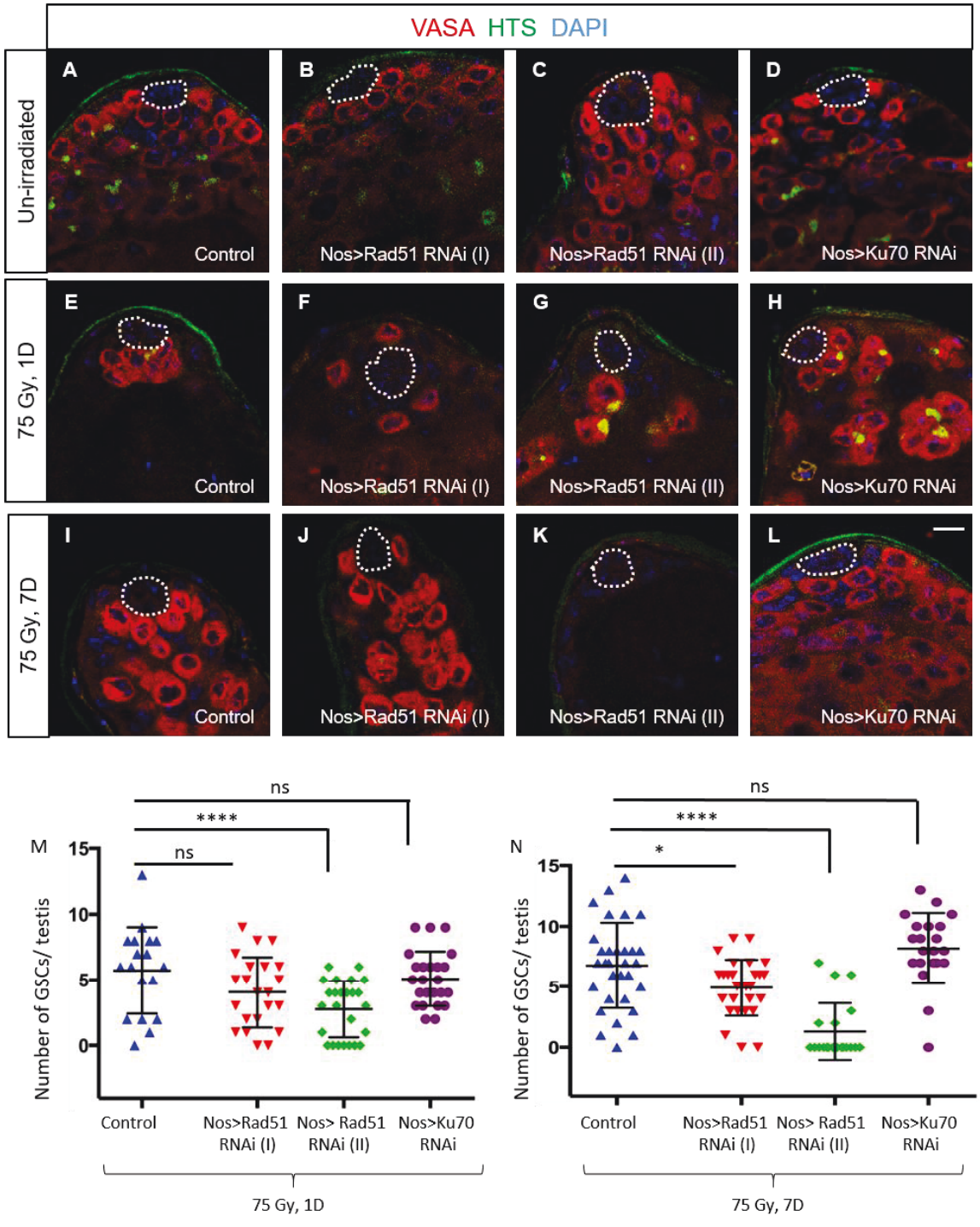

VASA HTS DAPI
Un-irradiated
75 Gy, 1D
75 Gy, 7D
A
B
C
D
E
F
G
H
I
J
K
L
Blm+/-
Blm-/-
Rad51+/-
Rad51 -/-
M
N
ns
****
Number of GSCs/ testis
N=18 N=13 N=12 N=11 N=18 N=23
N=23 N=26 N=21 N=24 N=20 N=39
Control Blm-/-
Control Rad51-/-
Un-irradiated
75 Gy, 1D
75 Gy, 7D

Figure 3.7


VASA HTS DAPI
Un-irradiated
75 Gy, 1D
75 Gy, 7D
A
B
C
D
E
F
G
H
I
J
K
L
Control
Nos>Rad51 RNAi (I)
Nos>Rad51 RNAi (II)
Nos>Ku70 RNAi
M
N
Number of GSCs/ testis
ns
****
*
Control
Nos>Rad51 RNAi (I)
Nos> Rad51 RNAi (II)
Nos>Ku70 RNAi
75 Gy, 1D
75 Gy, 7D

Supplementary Figures

Figure S3.1

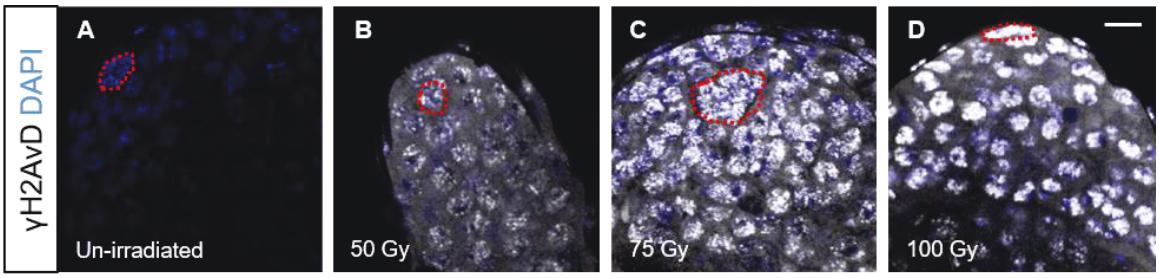


Figure S3.2

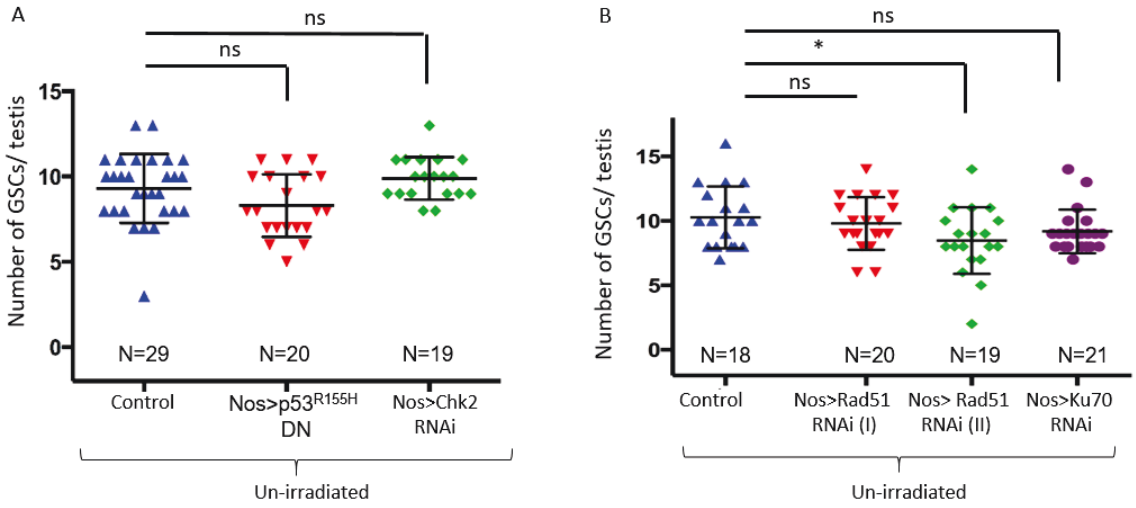

A
ns
ns
Number of GSCs/ testis
15
10
5
0
N=29
N=20
N=19
Control
Nos>p53R155H DN
Nos>Chk2 RNAi
Un-irradiated
B
ns
*
ns
Number of GSCs/ testis
15
10
5
0
N=18
N=20
N=19
N=21
Control
Nos>Rad51 RNAi (I)
Nos> Rad51 RNAi (II)
Nos>Ku70 RNAi
Un-irradiated

Figure S3.3

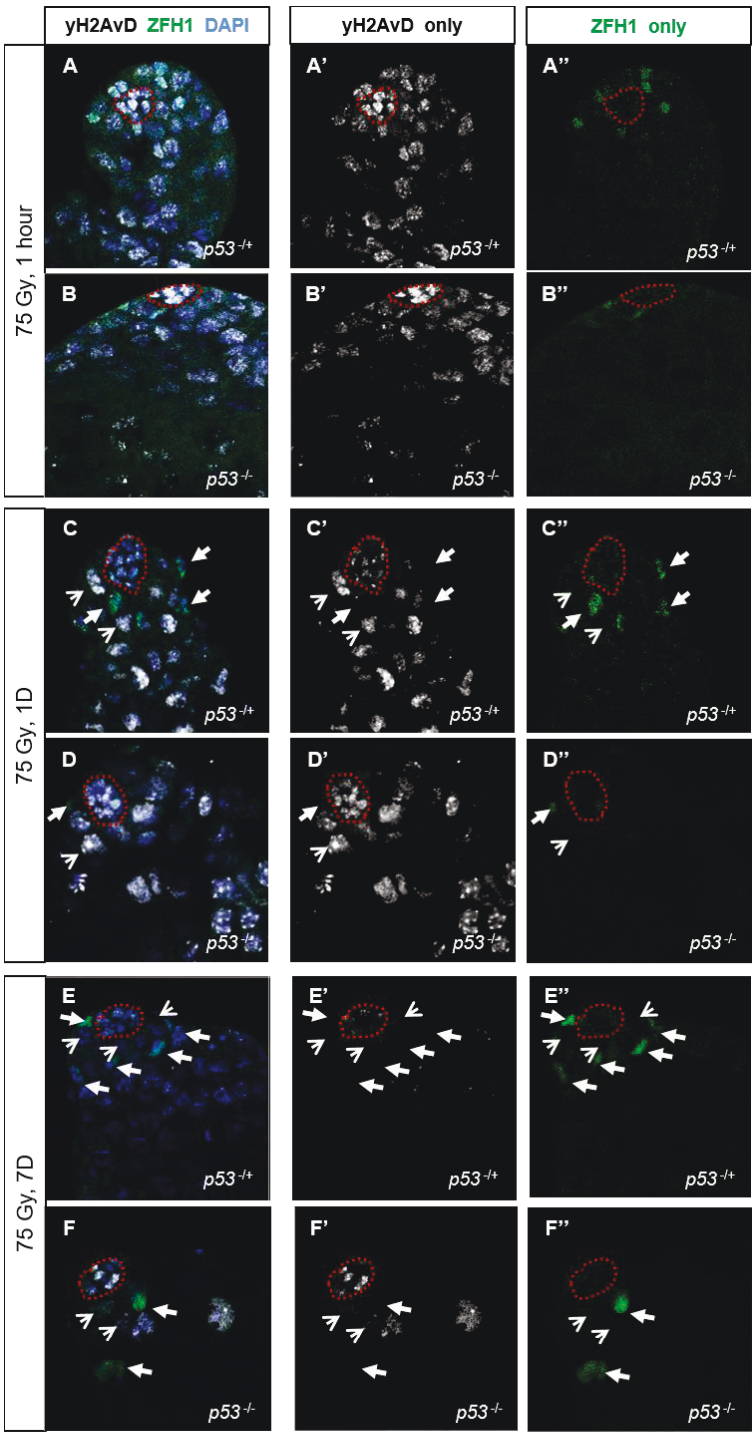
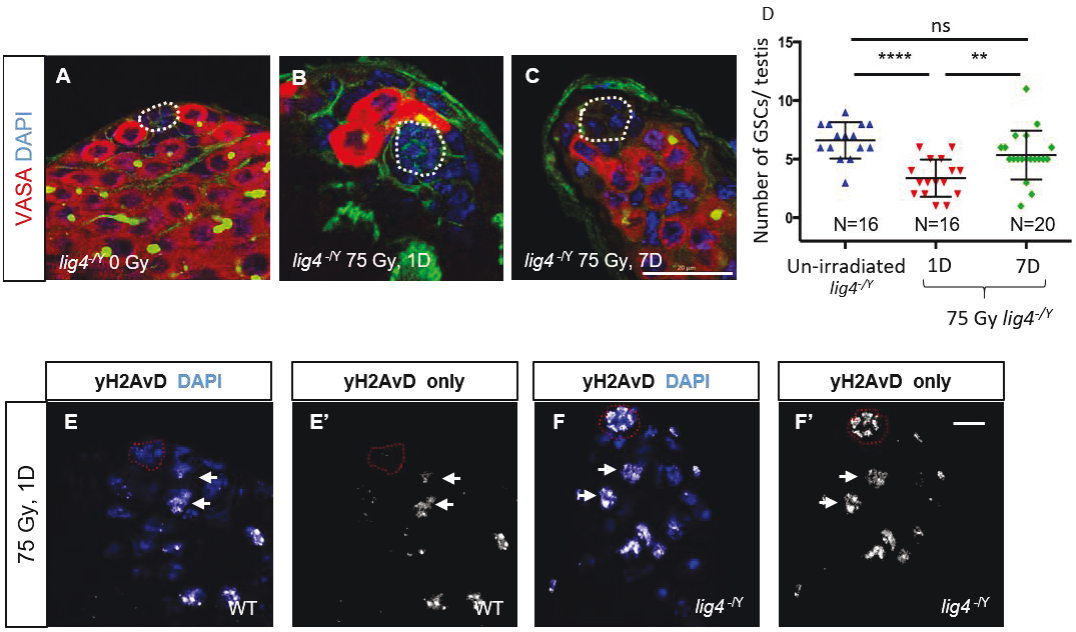

yH2AvD ZFH1 DAPI
yH2AvD only
ZFH1 only
75 Gy, 1 hour
75 Gy, 1D
75 Gy, 7D
A
A'
A''
B
B'
B''
C
C'
C''
D
D'
D''
E
E'
E''
F
F'
F''
p53 -/+
p53 -/-

Figure S3.4


VASA DAPI
A
B
C
lig4-/Y 0 Gy
lig4-/Y 75 Gy, 1D
lig4-/Y 75 Gy, 7D
D
ns
****
**
Number of GSCs/ testis
15
10
5
0
N=16
N=16
N=20
Un-irradiated
lig4-/Y
1D
7D
75 Gy lig4-/Y
yH2AvD DAPI
yH2AvD only
yH2AvD DAPI
yH2AvD only
75 Gy, 1D
E
E'
F
F'
WT
WT
lig4-/Y
lig4-/Y

Appendix-I

Data not included in other chapters

Figure legends

Figure A1. Mitochondria can be identified using Mito-GFP or Mito-tracker.

The experiment was done to specifically label mitochondria in fixed testis.

Fly husbandry: Genotype of flies dissected were Nanos Gal4 (VP16)> UAS mito-GFP (BDSC-8443) or ;Bam Gal4/ UAS mito-GFP (BDSC-8443). UAS mito-GFP/Cyo (BDSC-8442) shows similar expression pattern as ;;UAS mito-GFP (BDSC-8443)(data not shown) . Flies were crossed and grown at 25 ºC without $Gal80^{ts}$.0-4 days old flies were dissected, fixed and stained.

Database – TS2/Salman/Appendix data/mito project/mito GFP

(A -C) Confocal sections through the apex of testes immuno-stained with anti-Vasa antisera (marks germ cells, red), green fluorescent protein (GFP) (marks mitochondria) and DAPI (DNA, blue). Hub is outlined in white. Testes expressing GFP tagged with inner mitochondrial membrane targeting sequence in GSCs and immediate daughters (A) or in spermatogonia (B). GFP only segments are shown in A'-B'. Mitochondria are stained with the mitochondrial dye Mito-tracker in y w fly testes (red, 200 nM in Schneider's media) (see Appendix II) in C, C'. Scale bar 20 μm.

Mito-GFP seems to stain mitochondria robustly. Mito-tracker staining is erratic and not all testes in the tube get stained properly.

Figure A2. Mitochondrial damage in GSCs and immediate daughters but not in spermatogonia lead to germ cell loss.

The aim of the experiment was to understand the impact of mitochondrial DNA damage in GSCs and spermatogonia.

Fly husbandry: Flies of genotype Nanos Gal4 (VP16)>UAS mito-XhoI or Nanos Gal4 (VP16)> UAS mito-BglII (expression is in GSCs and immediate daughters) or Bam Gal4>UAS mito-XhoIor Bam Gal4>UAS mito-BglII (expression is in spermatogonia) were used. Both UAS mito-XhoI and UAS mito-BglII flies were kind gift of Dr. Hong Xu (Xu et al., 2008). No $Gal80^{ts}$ was used. Flies were crossed and grown at 25 ºC. 0-4 days old male progeny were dissected, fixed and stained.

Database – TS2/Salman/Appendix data/mito project/ mito XhoI or mito BglII

(A-B) Illustration of damage induced by mitochondria targeted XhoI or BglII (Xu et al., 2008). XhoI induces cuts in mitochondrial DNA leading to dysfunctional complex IV in inner mitochondrial membrane (A). BglII induces cuts in mitochondrial DNA leading to dysfunctional complex I in inner mitochondrial membrane (B).

(C-H) Confocal sections of testes immuno-stained with anti-Vasa antisera (marks germ cells, red), anti-Traffic Jam antisera (somatic cyst cells, green) (C-E) and DAPI (DNA, blue) under control conditions (C, F), expressing mtXhoI (D) or mtBglII (E) in GSCs and immediate daughters or expressing mtXhoI (G) or mtBglII (H) in spermatogonia. Hub is outlined in white.

All flies expressing mtXhoI (N=24 testes) or mtBglII (N=5 testes, testes were so small they were hard to identify) in GSCs and immediate daughters with Nanos Gal4 developmentally had extreme loss of germ cells by adulthood. All of the testes were small and not fully developed. However, expressing mtBglII or mtXhoI in spermatogonia did not show any phenotype and the testes were no different from Bam Gal 4 only controls.

Figure A3. Mitochondrial damage in spermatogonia may cause germ cell aggregates.

The aim of the experiment was to understand the impact of increased mitochondrial DNA damage in spermatogonia by shifting flies expressing mtXhoI or mtBglII to higher temperature.

Fly husbandry: UAS mito-BglII; Bam Gal4 or UAS mito-XhoI; Bam Gal4 were used for expression in spermatogonia. Flies were crossed and grown at 29 ºC. 0-4 days old male progeny were dissected, fixed and stained. A subset of flies was moved to 31 ºC for 7 days to further up-regulate the expression of mtXhoI or mtBglII. No $Gal80^{ts}$ was used.

Database – TS2/Salman/Appendix data/mito project/bam at 29 C

(A-F) Confocal sections of testes immuno-stained with anti-Vasa antisera (marks germ cells, red), anti-Hts antisera (1B1) (fusome, green) (C-E) and DAPI (DNA, blue) in controls (A, D), or expressing mtXhoI (B, E) or mtBglII (C, F) in spermaotgonia. The crosses were setup at 29 ºC (A-F) and were either dissected (A-

C) or moved to 31 ºC for 7 days (D-F) and dissected. Hub is outlined in white or marked with asterisk, germ cell aggregates are outlined in yellow.

(G-H) Histograms showing the percentage of testes with germ cell aggregates. Significant increase in testes with germ cell aggregates is seen when mtBglII but not mtXhoI is over-expressed (crosses grown at 29 ºC) (G) or when mtBglII or mtXhoI is over-expressed by shifting flies grown at 29 ºC to 31 ºC for 7 days. *** $p<0.001$, 2-tailed Fishers exact test.

(I-K) Confocal sections through the apex of testes immuno-stained with anti-Vasa (germ cells, red), anti-Phospho-histone 3 (PH3) (dividing cells, red), FasIII (hub and somatic membranes, green) and DAPI. Testes over-expressing mtBglII (J) or mtXhoI (K) (flies grown at 29 ºC) had germ cell aggregates with only some of them having PH3 stain compared to controls where all germ cell clusters undergo cell division simultaneously. Each figure is so oriented that the hub is towards the top of the image.

This experiment showed that spermatogonia do not die but form aggregates when mitochondria are impacted by increased expression of mtBglII or mtXhoI. These aggregates do not divide simultaneously, suggesting they are not a single germ cell cluster but aggregates of clusters; they could also be single clusters that have lost synchrony. However, since these clusters are also present in Bam Gal4 only controls, it is possible that the high temperature itself has some effect on germ cells in adult testis.

Figure A4. DNA damage can be induced specifically in germ cells using RNAi against spliceosome member proteins BugZ and Bub3.

The aim of the experiment was to induce DNA damage specifically in GSCs and their daughters without impacting neighboring cells.

Fly husbandry: UAS Dicer 2; Nanos Gal4 (NGT) (BDSC-25751) virgin females were crossed to ;; UAS BugZ RNAi (BDSC-27996, Valium 10, needs Dicer) or ;UAS Bub3 RNAi; (BDSC-40910) or ;; UAS Bub3 RNAi (BDSC-32989) at 25 ºC. No $Gal80^{ts}$ was used. 0-4 days old flies were dissected, fixed and stained. Expressing UAS Bub3 RNAi on II (BDSC-40910) led to loss of germ cells and adult testes were small and malformed (data not shown). I also tried to express UAS BugZ RNAi (BDSC-27996) in the hub using the E132 Gal4;;$Gal80^{ts}$ driver at 25 ºC or by growing the cross at 18 C and shifting to 29 ºC for 2 days. I did not see any increase in yH2AvD beyond the background level in any of these conditions for the hub.

Database – TS2/Salman/Appendix data/ BugZ and Bub3

(A-D) Confocal sections of testes immuno-stained with anti-yH2AvD antisera (marks DNA damage, red (A-B) or white (C-D)), anti-Hts (1B1) (fusome, green) (A-B) or anti-Traffic Jam antisera (somatic cyst cells, green) (C-D) and DAPI (DNA, blue) under control conditions (A, C), expressing RNAi against BugZ (B) or Bub3 (D) in GSCs and immediate daughters. Expression of RNAi against BugZ (B) or Bub3 (D) (Wan et al., 2015) shows increase in yH2AvD stains in GSCs and their immediate daughters (white arrows, A'-D'). No other phenotype is visible at this

time-point (0-4 days old flies). No other time points were assayed. All Z-section imaged at same gain. Hub is outlined in white.

Expression of Bub3 or BugZ RNAi can lead to targeted DNA damage. Staining with antisera raised against S9.6 (kind gift of Koshland lab, 1:1000) (Wan et al., 2015) to identify R-loops that would form due to improper splicing or antisera raised against BugZ (kind gift of Zheng lab, 1:100) (Wan et al., 2015) did not work (no immuno-stain was visible, data not shown).

Figure A5. Repair reporter 3 (Rr3) can indicate DNA damage repair pathway choice in germ and somatic cells in the testis.

The aim of the experiment was to use Rr3 to identify the pathway used to repair DNA damage in the cells of the testis.

Fly husbandry: ;Rr3/CyO; (gift from Schupbach lab) (Preston et al., 2006) females were crossed to either ;;HS-I-SceI (called 70I-SceI, BDSC-6935) or ;;Bam-I-SceI (gift of Huen lab) (Chan et al., 2011) (Number 31 in my fly stock collection). Crosses were raised at 25 ºC. Adult males of age indicated in the figure of genotype Rr3; HS-I-SceI were heat shocked for 1 hour at 37 ºC. After heat shock, the males were collected and crossed to yw virgin females (up to 10 males with twice the number of females per bottle, cross was tossed after one week). The progeny of these flies were collected and frozen at -20 ºC (freezing is not essential, I did it to make it easier to store flies and analyze later). The frozen flies were then analyzed for Ds-Red expression using Night-Sea system or their genomic DNA was extracted, Rr3 region amplified through PCR and then run on a gel. Bam-I-SceI containing flies did not

need to be heat-shocked because I-SceI expression in them is regulated by *bam* promoter. Male progeny of genotype Rr3; Bam I-SceI were crossed to virgin females and their progeny analyzed similar to above.

Database – TS2/Salman/Appendix data/ Rr3

(A) Illustration indicating how Rr3 can be used to find repair pathway choice in germ cells (adapted from (Preston et al., 2006)). Induction of the mega-endonuclease I-SceI causes a single double strand break in the Rr3 locus (in red). This locus can then be repaired using Homologous repair (HR), Non homologous end joining (NHEJ) or single strand annealing (SSA). HR only occurs if a homologous chromosome containing the modified Rr3 locus (lacking the cut site for I-SceI) is present. DNA damage repair in the germ cells can be detected in the next generation in the following manner: SSA is indicated by a red fly due to Ds-red expression from Actin 5C promoter. NHEJ and HR can be identified by whole fly genome PCR (see appendix III for PCR conditions) where double bands indicated HR and single band NHEJ.

(B) Figure indicating the outcomes of the HR, NHEJ or SSA repair pathways using the Rr3 system. SSA yields a red fly progeny, NHEJ yields a single band shifted from its expected 466 bp size and HR is shown by two bands – one at 466 bp and other and 232 bp.

(C-D) Confocal sections of testes immuno-stained with anti-DsRed antisera (marks SSA repair, red), anti-Traffic Jam (somatic cyst cells, green) and DAPI (DNA, blue). I-SceI was induced by heat shock for 1 hour at 37 ºC in 14-17d old flies (4-7d post

ecclosion) (C) or 33-36d old flies (23-26d post ecclosion) (D). DsRed stain in the Cyst cells shows that these cells have undergone DNA damage repair using the SSA pathway. Hub is outlined in white.

(E) Table showing outcomes of DNA repair in germ cells in flies using two different promoters (Bam and HS) to induce I-SceI expression or Bam promoter to specifically induce I-SceI in spermatogonia only. The table shows number of offspring (out of total offspring) that are derived from germ cells repaired by SSA or NHEJ. The repair was assayed in flies lacking the homologous region for Rr3 cut site, thereby blocking HR from occurring. SSA was assayed based on red progeny and NHEJ from PCR of whole genome isolated from progeny flies where DNA damage was induced.

Rr3 reporter system can be used to analyze DNA damage repair but it seems to work best for assaying repair by SSA. While gel electrophoresis analysis works only for major changes in band size induced by insertion or deletion in the Rr3 locus due to NHEJ. Minor changes like addition or deletion of few nucleotides is not clearly discernable from the gel. Additionally, both HS-I-SceI and Bam-I-SceI did not have 100% cutting efficiency. A lot of the progeny did not show any change at the Rr3 cut site upon sequencing (data not shown). Since HR was abolished in these flies, it is possible that the cut sites were repaired accurately in these cases by NHEJ or no cut was induced.

Figure A6. p53-GFP reporter shows activation of p53 in response to DNA damage

The aim of the experiment was to assay activation of p53 in response to radiation-induced DNA damage.

Fly husbandry: 0-4 day old, ;;p53-GFP nuclear localization signal (NLS) (gift from Shinya in Drummond-Barbosa lab) (Wylie et al., 2014) flies were irradiated at 100 Gy and dissected as stated below.

Database – TS2/Salman/Appendix data/ p53-GFP reporter

(A) Illustration from (Wylie et al., 2014) shows transcriptional activation of GFP in response to stress like radiation. The p53 reporter has been created by using the p53 binding site from the Reaper promoter region and fusing it to a GFP coding sequence. When p53 is activated, it induces transcription of GFP.

(B) Histogram showing % of testes with GSCs marked with GFP showing activation of p53, at three time points- 2hr, 1day and 1.5 day after exposure to 100 Gy radiation.

(C-E) Confocal sections of testes immuno-stained with anti-Vasa antisera (germ cells, red), anti-GFP (p53 activation, green) and DAPI (DNA, blue). p53 was activated by 100 Gy radiation. While GFP was not visible 2 hr post radiation exposure (C, C'), robust expression of GFP was visible in GSCs as well as their daughters at 1d (D, D') or 1.5 d post (E, E') 100 Gy radiation.

P53-GFP NLS reporter shows p53 activity robustly and in the hub, GSCs and spermatogonia and the somatic cyst cells after irradiation.

Figures

Figure A1

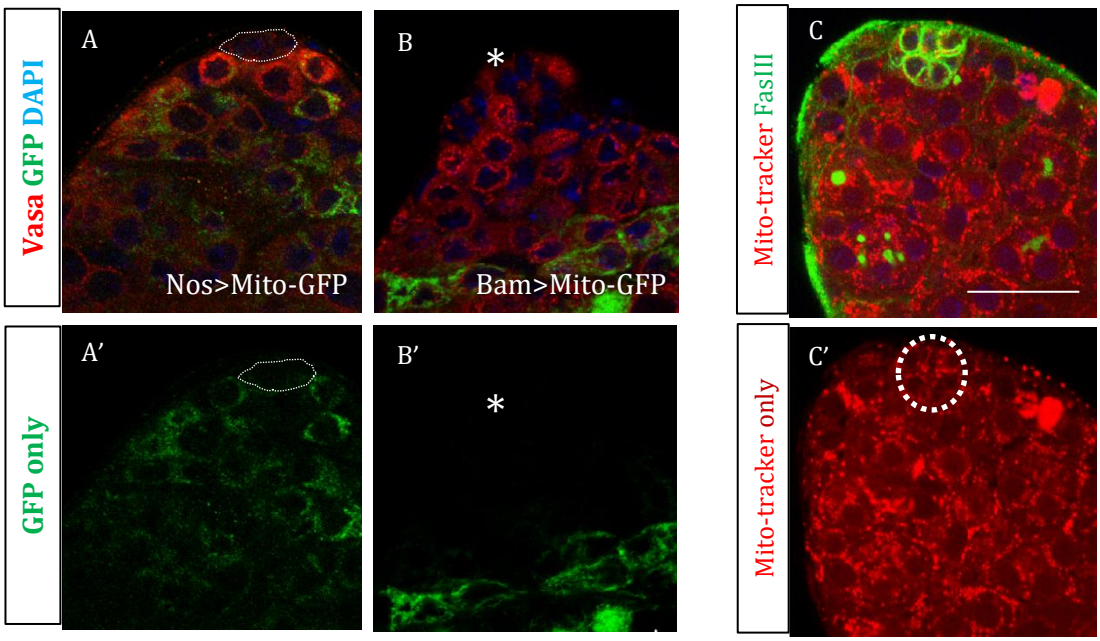


Figure A2

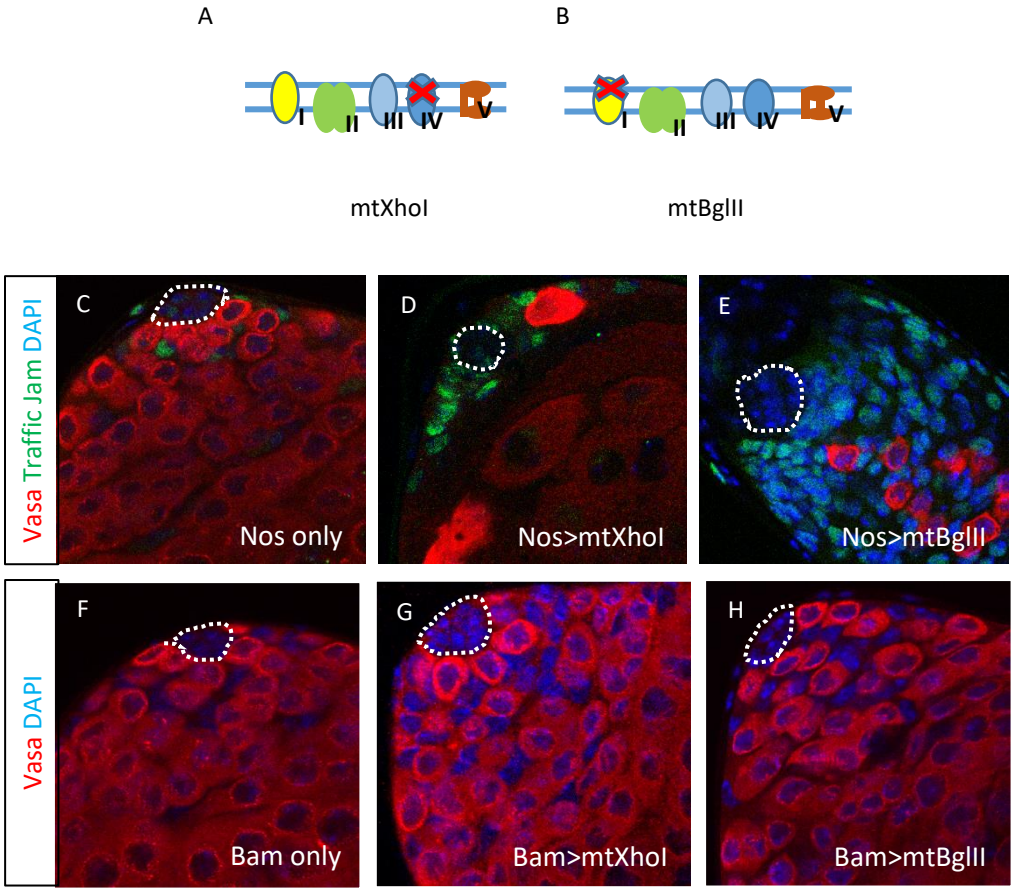

A
B
I
II
III
IV
V
mtXhoI
mtBglII
Vasa Traffic Jam DAPI
C
Nos only
D
Nos>mtXhoI
E
Nos>mtBglII
Vasa DAPI
F
Bam only
G
Bam>mtXhoI
H
Bam>mtBglII

Figure A3

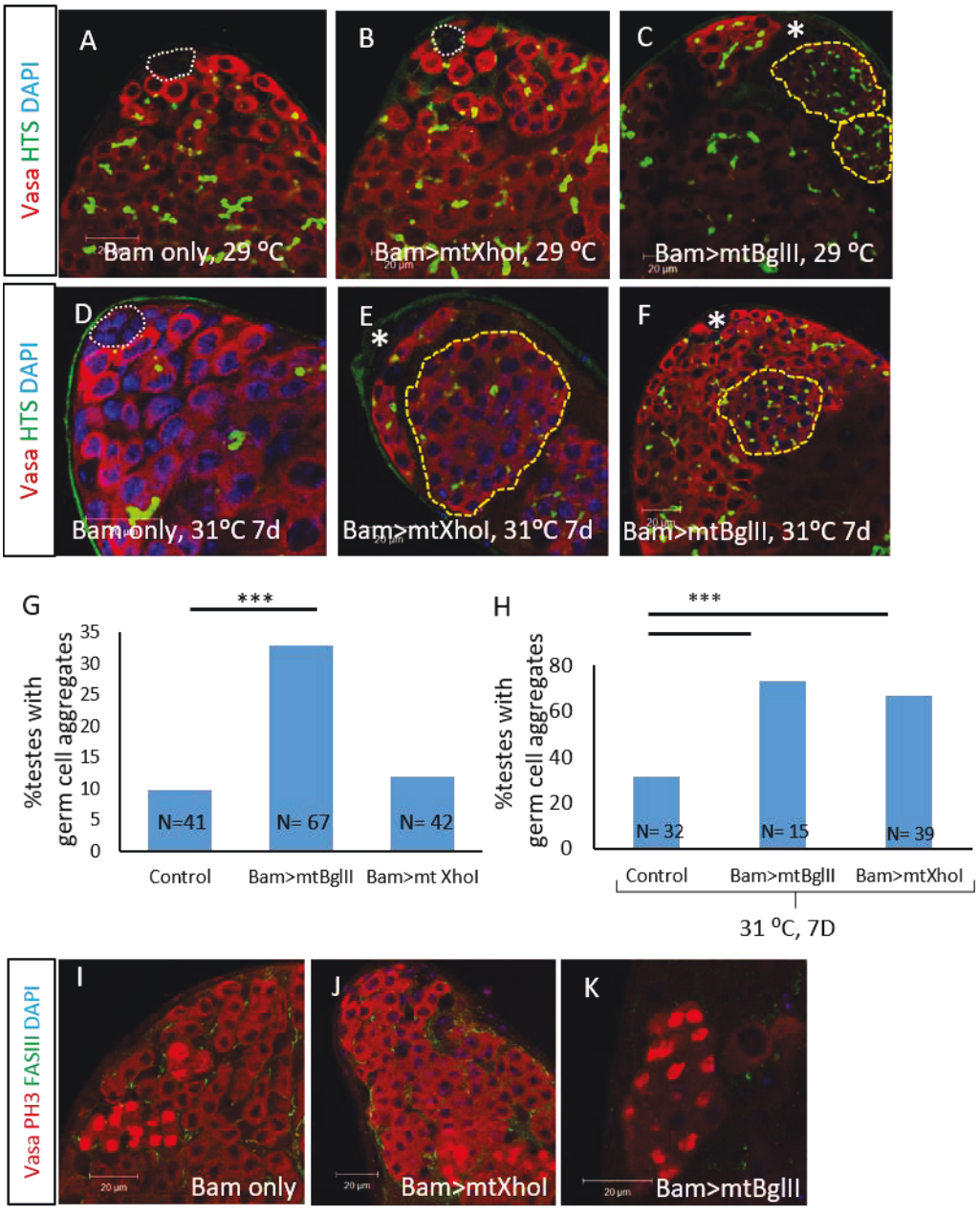

Vasa HTS DAPI
A
Bam only, 29 °C
B
Bam>mtXhoI, 29 °C
C
Bam>mtBglII, 29 °C
Vasa HTS DAPI
D
Bam only, 31°C 7d
E
Bam>mtXhoI, 31°C 7d
F
Bam>mtBglII, 31°C 7d
G
***
%testes with germ cell aggregates
35
30
25
20
15
10
5
0
N=41
N= 67
N= 42
Control
Bam>mtBglII
Bam>mt XhoI
H
***
%testes with germ cell aggregates
80
60
40
20
0
N= 32
N= 15
N= 39
Control
Bam>mtBglII
Bam>mtXhoI
31 °C, 7D
Vasa PH3 FASIII DAPI
I
Bam only
J
Bam>mtXhoI
K
Bam>mtBglII

Figure A4

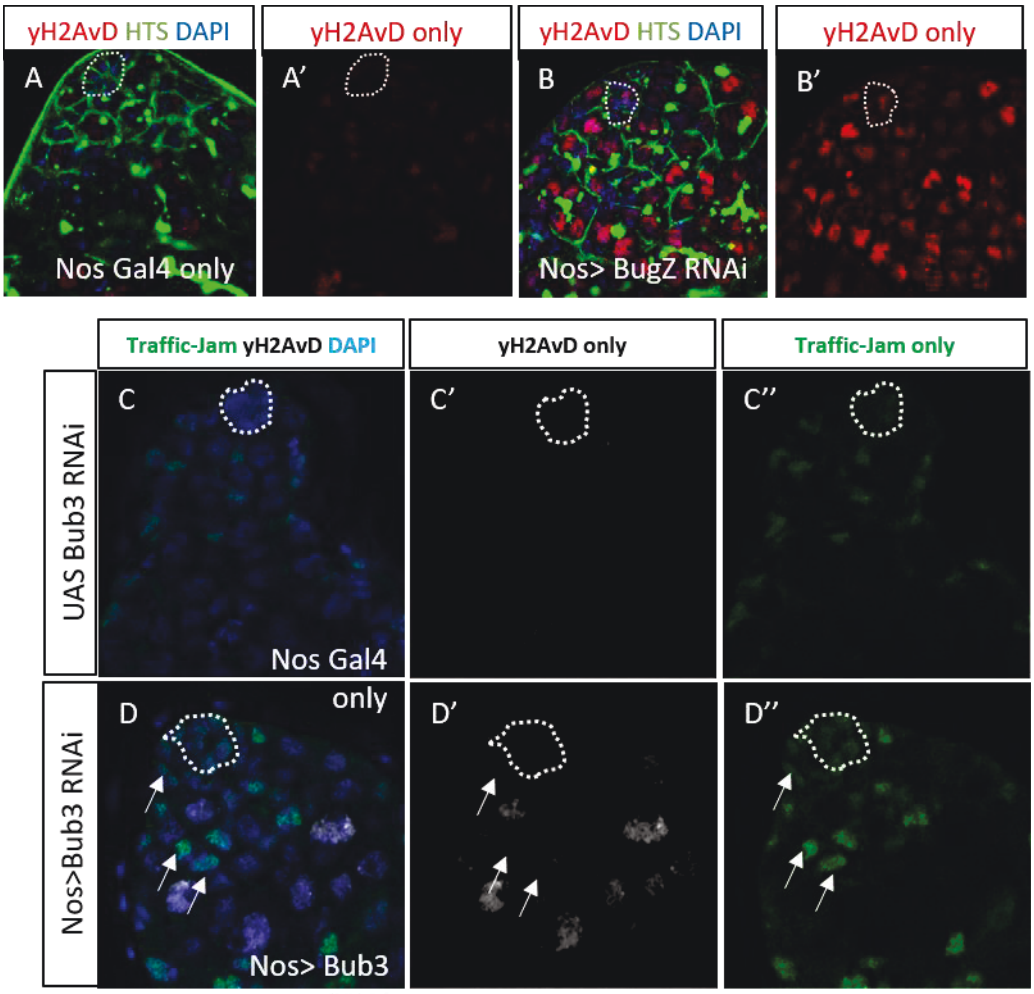
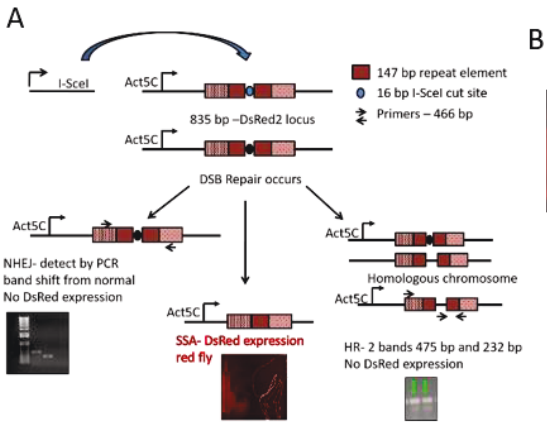
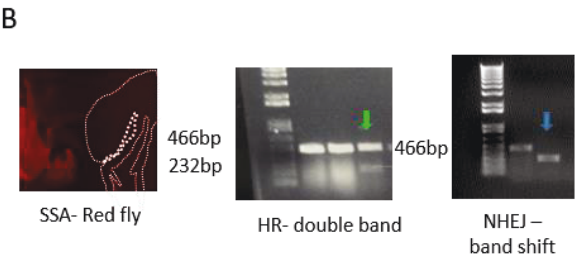
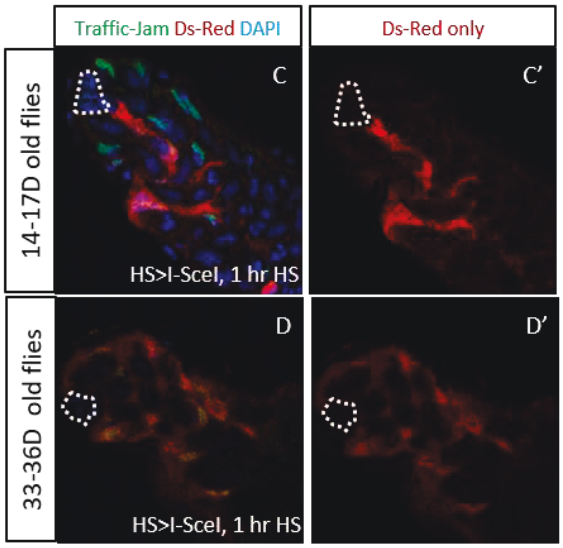

yH2AvD HTS DAPI
yH2AvD only
yH2AvD HTS DAPI
yH2AvD only
A
A'
B
B'
Nos Gal4 only
Nos> BugZ RNAi
Traffic-Jam yH2AvD DAPI
yH2AvD only
Traffic-Jam only
UAS Bub3 RNAi
C
C'
C''
Nos Gal4 only
Nos>Bub3 RNAi
D
D'
D''
Nos> Bub3 RNAi

Figure A5



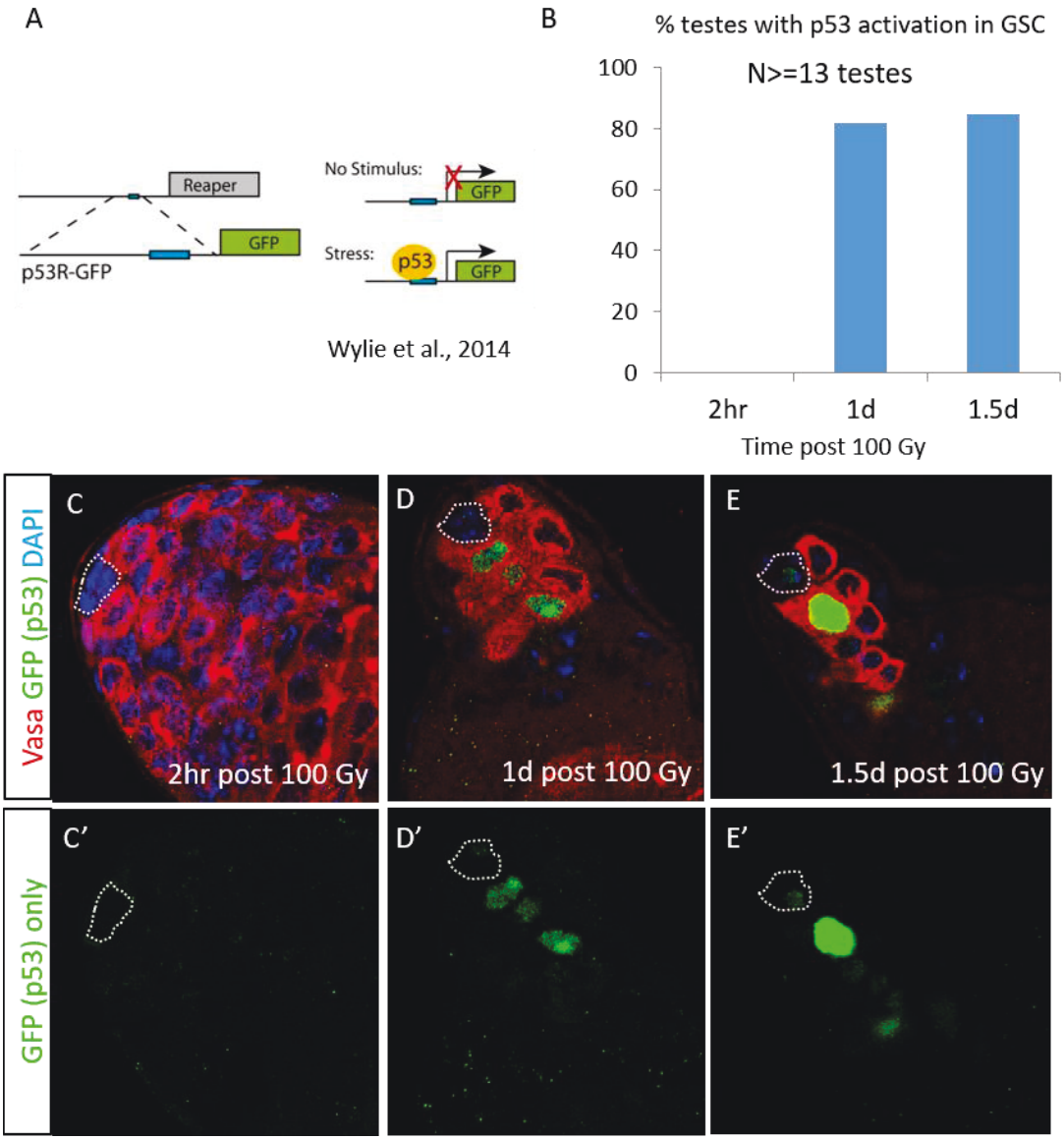




E

Father's age	I-SceI driver	SSA	NHEJ
0-4 d	HS (Germ cells)	106/189 (56%)	83/189 (44%)
0-4 d	Bam (Spermatogonia)	174/297 (58.5%)	123/297 (41.5%)
7-11 d	Bam (Spermatogonia)	109/186 (58.6%)	77/186 (41.4%)

Figure A6


A
Reaper
GFP
p53R-GFP
No Stimulus:
GFP
Stress:
p53
GFP
Wylie et al., 2014
B
% testes with p53 activation in GSC
N>=13 testes
100
80
60
40
20
0
2hr
1d
1.5d
Time post 100 Gy
Vasa GFP (p53) DAPI
C
2hr post 100 Gy
D
1d post 100 Gy
E
1.5d post 100 Gy
GFP (p53) only
C'
D'
E'

Appendix –II

Mito-tracker staining protocol for fly testis

This protocol is adapted from (Metzendorf and Lind, 2010) and can be used to stain testes in ex-vivo culture (in a dish) or in tubes where testes are still attached to cuticles. Use the dish for live imaging; otherwise, tubes are easier (since they don't require the poly-lysine step).

1. If using the dish, add poly-lysine (same as used for live imaging) to the dish and leave at room temperature (RT) for at least 1 hour (overnight is ok). Remove the poly lysine and store at 4 ºC (it can be reused for up to 1 week). Wash the dish 2-3 times with water and leave water in it to avoid it from drying.
2. Prepare 200 nM mito-tracker in Schneiders media – add 0.1 µl of mito-tracker red (powder dissolved in 94 µl DMSO to concentration of 1mM, Cell signaling technology), 10 µl of insulin, 2.75 µl of Penn-strep and 75 µl of fetal bovine serum to 412.25 µl of Schneiders. 500 µl of this solution is used per tube/ dish.
3. Dissect flies in 1X Ringers solution, leave the cuticle on if using tube or take the entire testis out of the fly, if using a dish.
4. Move the dissected testes to the tube or dish. Testes should not dry out, so move them along with some 1X Ringers solution.
5. Remove 1X Ringers solution completely and add the mito-tracker solution prepared in step 2 to the tube/ dish. Alternatively, testes can be dissected and placed directly into the mito-tracker solution.

6. **Important** (and different from the published protocol): Cover the dish or tube in aluminum foil and let it sit over night at RT. Do not place the tube on nutator.
7. Wash with 1X phosphate buffered saline (PBS) for 10 minute.
8. Fix with 3.7% formaldehyde (115 µl of 16% formaldehyde in 385 µl 1X PBS) for 15 minutes. Note: This fixation and the following staining / washing steps differ from our standard protocol.
9. Wash 2 times in 1X PBS for 10 minutes each.
10. Wash 1 time in 1X PBS containing 0.3% Triton-X (PBX) for 10 minutes.
11. Add primary antibody in block (2% normal goat serum in 1X PBX with 0.02% bovine serum albumin and 0.02% sodium azide) for overnight staining at 4˚C (at this point you can directly proceed to step 12 if no primary staining is desired).
12. Wash twice in 1X PBS for 10 minutes each.
13. Add secondary antibody and DAPI in block without normal goat serum for 3-4 hours at RT or overnight at 4 ºC.
14. Wash twice in 1X PBS for 10 minutes each.
15. Mount in Vectashield.

Appendix –III

Polymerase chain reaction (PCR) method for Rr3 experiment assaying for NHEJ only

	Final Concentration	Volume (µl)
GoTaq Master mix	1X	12.5
Genomic DNA	<250 ng	1.5
Primer Red01	0.25 µM	0.625
Primer Red06	0.25 µM	0.625
ddH2O (nuclease free)	-	9.75

PCR cycles

95 ºC @ 2 min

95 ºC @ 30 sec
61 ºC @ 1 min
72 ºC @ 1 min
} X 25 Cycles

72 ºC @ 5 min

Hold @ 4 ºC

Primers

Red01: GCCTCCTCCGAGAACGTCATCAC

EJ3: CCGGCTAGGGATACGGCCGGG

Red06: GCCGTCCTCGAAGTTCATCA

Red01 primer is to be used for both HR and NHEJ sequencing. Red06 is specific to NHEJ and EJ3 specific to HR. If doing an Rr3 assay where both NHEJ and HR outcome are possible one should use all three primers with double the volume of Red01 than Red06 and EJ3. For further details please check (Preston et al., 2006).

Appendix –IV

Making transgenic fly containing the Traffic light reporter (TLR) to assay DNA damage repair (NHEJ or HR only) in testis stem cells

Aim: We would need to make two flies one with the TLR construct and one with the donor construct (d14-GFP) to assay HR. To make the TLR containing fly, TLR locus would be extracted from the fragment from the plasmid (Addgene-31482) (Certo et al., 2011) in the fly vector pDTubHA4Ca [attP] (kind gift of Rincon-Limas lab) (Zhang et al., 2013). Once the plasmid is ready it would be inserted in appropriate fly (I hadn't decided on which fly to use) to yield Tub-TLR genotype. To make the d14-GFP fly, we would need to insert d14 GFP (Addgene -31475) (Certo et al., 2011) in the fly vector pDTubHA4Ca [attP].

Procedure: The procedure is adapted from Addgene protocol for PCR based cloning (https://www.addgene.org/protocols/pcr-cloning/). I tried to digest the TLR plasmid (31482) or d14 plasmid (31475) with EcoRI and XhoI. The digested segment would then be pasted into the pDTubHA4Ca [attP] plasmid, cut with EcoRI and XhoI. The primers for PCR based cloning are given in the table below.

Result: In my experiments I sequenced all the three plasmids for the proper sites and each of them had the relevant cut sites and the gene sequences. I also cut the fragment of interest from the TLR plasmid and the d14 plasmid successfully (based on band size, no sequencing). However, I was not able to ligate the fragment into the recipient plasmid.

Location- Plasmids are in a box labeled "TLR" in Bench 1 freezer. The restriction digest data is in lab notebook #3 and at TS2/Salman/TLR

Gene copied	EcoRI (5')	XhoI (3')
TLR	TAAGCAGAATTCATGGTGAG CAAGGGCGAG	TGCTTACTCGAGTCACTTGTAC AGCTCGTC
d14GFP	TAAGCAGAATTCATGGTGAG CAAGGGCGAG	TGCTTACTCGAGTCACACGAA CTCCAGCAG

TLR length of fragment is 1513 bp

d14 GFP length of fragment is 677 bp

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Curriculum Vitae

The Johns Hopkin University School of Medicine

Salman Hasan April 2nd 2017

Born: July 7th 1988 at Lucknow, India

Educational History

Ph.D. expected	2017	Program in Biochemistry, Cell and Molecular biology Mentor: Erika Matunis, PhD	The Johns Hopkins University, School of Medicine, Baltimore
Integrated B.S. - M.S.	2011	Biological Sciences Mentor: Chanchal Das Gupta, PhD	Indian Institute of Science Education and Research, Kolkata

Professional Experience

Research rotation	2012-2012	Lab of Michael Catarina, PhD, Johns Hopkins University
Research rotation	2011-2011	Lab of Jennifer Pluznick, PhD, Johns Hopkins University

Summer research fellow	2010-2010	Lab of Samuel Butcher, PhD, University of Wisconsin-Madison
Summer research fellow	2009-2009	Lab of N.G. Prasad, PhD, IISER Mohali

Scholarships. Fellowships

Hay fellowship	2014-2015	Awarded by the Dept. of Cell Biology, Johns Hopkins University, School of Medicine. Includes health insurance, graduate school dues and stipend
Khorana fellowship	2010-2010	Awarded by the Indo-U.S. science and technology forum, includes stipend, research support for summer

Publications

Hasan, S., Hetie, P., Matunis, E.L., (2015). Niche signaling promotes stem cell survival in the Drosophila testis via the JAK-STAT target DIAP1. Dev. Biol. 404, 27–39. PMCID: PMC4469572

Das, D., Samanta, D., **Hasan, S.**, Das, A., Bhattacharya, A., Dasgupta, S., Chakrabarti, A., Ghorai, P., Das Gupta, C., (2012). Identical RNA-protein interactions in vivo and in vitro and a scheme of folding the newly synthesized proteins by ribosomes. J. Biol. Chem. PMCID: PMC3481345

Posters

Hasan, S., Hetie, P., Matunis, E.L., (2015). Niche signaling promotes stem cell survival in the Drosophila testis via the JAK-STAT target DIAP1. Annual Drosophila Research Conference, Genetics Society of America, Chicago, March 2015.

Hasan, S., Hetie, P., Matunis, E.L., (2015).Regulation of stem cell survival under stress-induced conditions in the Drosophila testis niche. Annual Drosophila Research Conference, Genetics Society of America, San Diego, March 2014.

Service and leadership

2016-2017	Graduate student liaison for Gertrude Stein Society
2015-2016	Organized weekly student seminar series titled “Lewis Talks” for Dept. of Cell Biology, Johns Hopkins University School of Medicine.
2014-2015	Volunteer, Thread – program for helping high school students with classwork